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THE PHILIPPINE
JOURNAL OF SCIENCE

VOL. 44 JANUARY–FEBRUARY, 1931 NOS. 1–2

EXPERIMENTAL STUDIES OF DENGUE[1]

By JAMES STEVENS SIMMONS, PRESIDENT
Major, Medical Corps, United States Army
JOE H. ST. JOHN, MEMBER
Major, Medical Corps, United States Army
and
FRANCOIS H. K. REYNOLDS, MEMBER
Captain, Veterinary Corps, United States Army

THREE PLATES AND SIXTY-SEVEN TEXT FIGURES

INTRODUCTION

During the past thirty years the Medical Department of the United States Army has been actively interested not only in dengue but also in the similarly transmitted and more formidable disease, yellow fever. This interest, which was due, primarily, to the appalling mortality caused by yellow fever among our armed forces during the Spanish-American War and, later, to the extremely high morbidity rates for dengue and other infections among troops stationed in the Philippines, led to the formation of boards of medical officers, one in Cuba and another in the Philippine Islands, for the investigation of tropical diseases. The fundamental discoveries concerning the mosquito transmission of yellow fever, contributed by the board working in Cuba with Maj. Walter Reed in 1900, led to the almost complete eradication of that disease.

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[1] From the United States Army Medical Department Research Board, Bureau of Science, Manila, Philippine Islands. This article, with an appendix, consisting of protocols of the mosquitoes and the human volunteers, will be reprinted as a Bureau of Science monograph.

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The other board, appointed in 1900, for the investigation of tropical diseases as they exist in the Philippines, was dissolved in 1902, reformed in 1906, and after a period of inactivity from 1914 to 1922, was again reconvened and called the United States Army Medical Department Research Board. During this time succeeding members of the Philippine boards made valuable contributions to the knowledge of many of the important tropical diseases, including dengue. Ashburn and Craig, while working on this board in 1907, brought out certain fundamental information concerning the etiology, epidemiology, transmission, symptomatology, diagnosis, prophylaxis, and treatment of the disease. They reported the transmission of dengue experimentally to one of four volunteers with *Culex quinquefasciatus,* were the first to demonstrate the infective virus in the circulating blood of dengue patients, and were also first to establish the fact that the virus of dengue belongs to the group of so-called filterable viruses. In 1922 dengue was again made the subject of an extensive and important investigation by Siler, Hall, and Hitchens, while members of this board. These workers not only confirmed the fact that dengue is transmitted by *Aëdes ægypti,* but also determined the period of infectivity of dengue patients, the period required for development of the virus in *A. ægypti,* and the period during which infective *A. ægypti* are capable of transmitting dengue. They were unable to transmit dengue with *Culex quinquefasciatus.* Their comprehensive report on the history, epidemiology, mechanism of transmission, etiology, clinical manifestations, immunity, and prevention of dengue furnishes a most important source of information concerning the disease. Schule, working on the board in 1928, continued these investigations and confirmed the work of Siler, Hall, and Hitchens regarding the period of time that must elapse between the infectious feeding and the transmission of virus to man by *A. ægypti,* and showed that under certain conditions this period may be as short as eight days.

In 1928 the authors of the present report selected dengue as a subject for further study. The more important of the problems considered had to do with (*a*) the epidemiology of dengue; (*b*) the nature of the virus, its identification, size, filterability, appearance, virulence, viability in vitro, and its possible growth in culture media; (*c*) the possibility of transmitting dengue to man by insects other than *A. ægypti;* (*d*) the possibility of transmission of dengue from infected to normal insects; (*e*) search for a lower animal susceptible to dengue which would be suitable for use, instead of man, in diagnostic and experimental work; (*f*) search for improved methods of diagnosis by studies of the leukocyte curves and serological reactions of patients; (*g*) further studies of the immunity to dengue of man and lower animals; (*h*) observations on the therapeutic value of immune sera; and (*i*) the investigation of a prophylactic vaccine.

The results, even of negative observations, are submitted in detail, as it is believed that the data brought out should not only add certain fundamental information to the present knowledge concerning dengue, but may also prove of assistance to those engaged in experimental investigations of both dengue and yellow fever.

EPIDEMIOLOGY OF DENGUE IN THE PHILIPPINE ISLANDS

Although dengue has existed in the Philippine Islands for many years, there is no reliable information concerning the incidence of the disease or its spread among the native and white civilian inhabitants. Factors that have made the collection of such data impossible are (*a*) the failure of a large proportion of the native population to seek competent medical attention; (*b*) the difficulty experienced even by well-trained physicians in differentiating mild or atypical dengue from other acute febrile conditions, and (*c*) the fact that dengue is not considered a reportable disease by the Philippine Health Service.

Therefore, the epidemiological studies of dengue made in the Philippines—including those made by Ashburn and Craig(1) in 1907; by Siler, Hall, and Hitchens(2) in 1924; and those of the present board—have been limited mainly to the disease as it occurs in military personnel, and have been based either on investigations made at Army posts or on official records in the office of the Surgeon General, United States Army, and of the Surgeon, Philippine Department. As Siler, Hall, and Hitchens have already published a comprehensive statistical analysis of dengue in the Philippines for the period 1902 to 1924, certain of their figures have been used along with additional official records for the period 1924 to 1929 in order to extend their studies to the present time.

Dengue in the United States Army.—Since the first American troops arrived in the Philippines, dengue, because of its high morbidity and the resultant loss of time from hospitalization, has been a source of considerable economic loss to the Army.

A study of the geographical distribution of dengue among American soldiers shows that its importance to the Army as a whole is due almost entirely to its high incidence in the Philippine Islands. The annual dengue admission rates per thousand for white enlisted men serving in different countries during 1928, as indicated in fig. 1, were as follows: (*a*) The entire Army, 6.84; (*b*) the Army in the United States, 0.02; (*c*) the Army in Hawaii, 0; the Army in Panama, 0.5; and (*d*) the

Army in the Philippine Islands, 177.06. Of the seven hundred, sixty-two cases of dengue reported for white enlisted men in the entire United States Army during the year 1929, 97 per cent occurred in the Philippines. With minor variations these figures give a fairly accurate picture of the geographical distribution of the disease among American troops during previous years and indicate the great prevalence of dengue in the Philippine Islands.

Dengue in the Philippine Islands.—During the past twenty-seven years, as indicated in fig. 2, the annual admission rates from dengue have shown no sustained improvement, while during the same period the rates for all diseases considered together have decreased, indicating that with the exception of dengue there has been an improvement in the general health. In 1924 it was estimated by Siler, Hall, and Hitchens that during the twenty-five previous years dengue had been responsible for an average loss to the Army of 7,715 days per year; since 1924 the time lost has amounted to 1,978 days for 1925, 3,119 days for 1926, 5,450 days for 1927, and 5,226 days for 1928, an average loss per year of 3,943 days.

Distribution by Army posts.—The incidence of dengue differs strikingly at the various Army posts in the Philippines, as is indicated in fig. 3. Camp John Hay, which is situated in the mountains at an elevation of about 5,000 feet, has always been free from the disease, while the lowland posts in or near Manila have relatively high admission rates.

Camp John Hay is situated in the mountains of Luzon about 180 miles north of Manila at an elevation of approximately 5,000 feet. During 1928 the mean strength for white enlisted men was only 23, and for native soldiers 202. The temperature range for that year was as follows: Minimum, 12.5° C.; maximum, 29° C.; mean, 18° C.; and the annual rainfall was 178.24 inches. According to the

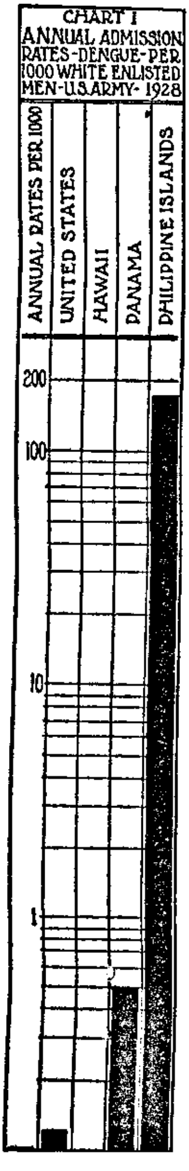

FIG. 1. Annual dengue admission rates per thousand white enlisted men in the United States Army, 1928.

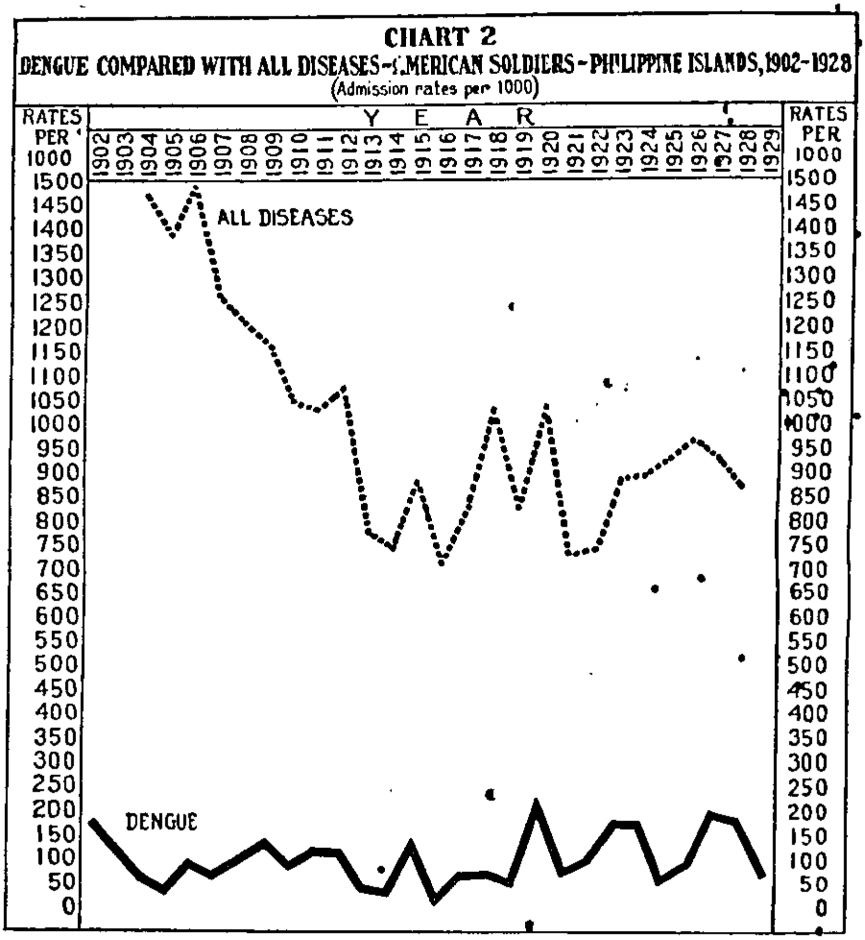

FIG. 2. Dengue compared with all other diseases contracted by American soldiers in the Philippine Islands, 1902–1928.

available information *Aëdes* mosquitoes have not been found there, although several species of *Anopheles* and *Culex* were collected in large numbers during April, 1929. It is claimed that dengue does not occur among the civilians living in the near-by City of Baguio; while the official records show that the disease does not occur either in the white or native soldiers of the military post. In this connection it is of interest to note that recently it has been shown experimentally by the authors that native Ifugaos living in this dengue-free post are extremely susceptible to dengue. Also that one native seen in April, 1928, had contracted the disease during a stay at Fort William

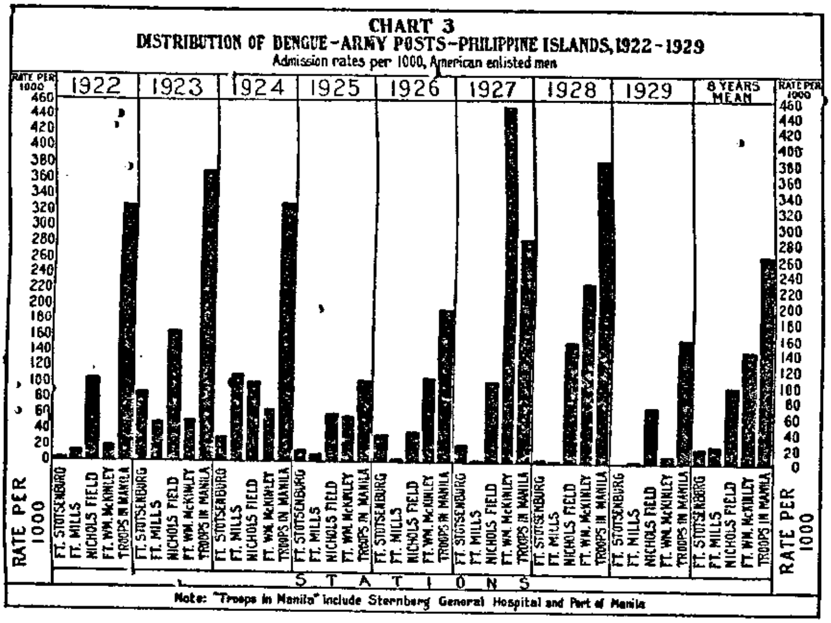

FIG. 3. Distribution of dengue among Army posts in the Philippine Islands, 1922-1929.

McKinley and had returned to Baguio during the second day of typical dengue. The high altitude and the resultant low temperature at Camp John Hay are probably responsible for the apparent freedom of that post from dengue.

Fort Stotsenburg, at an elevation of about 680 feet, is situated in Pampanga Province, 66 miles northwest of Manila, on a sloping sandy plain from which the reservation extends into mountainous country. The temperature range during 1927 was, average maximum, 32.5° C.; average minimum, 21.5° C.; with a daily variation of nearly 13.5° C. The average annual rainfall was 108 inches for the period 1916 to 1926, and the relative humidity for twelve months in 1927 was 91. There is no large city near this post, but several native barrios are located in the vicinity. The average yearly strength for American troops at Fort Stotsenburg has been as follows: 728 in 1922, 238 in 1923, 212 in 1924, 198 in 1925, 200 in 1926, 214 in 1927, 299 in 1928, and 190 in 1929.

The relatively low dengue admission rates for Fort Stotsenburg, indicated in fig. 4, have no doubt been due mainly to the isolation of the post and the active mosquito-control work which has been carried on there for several years.

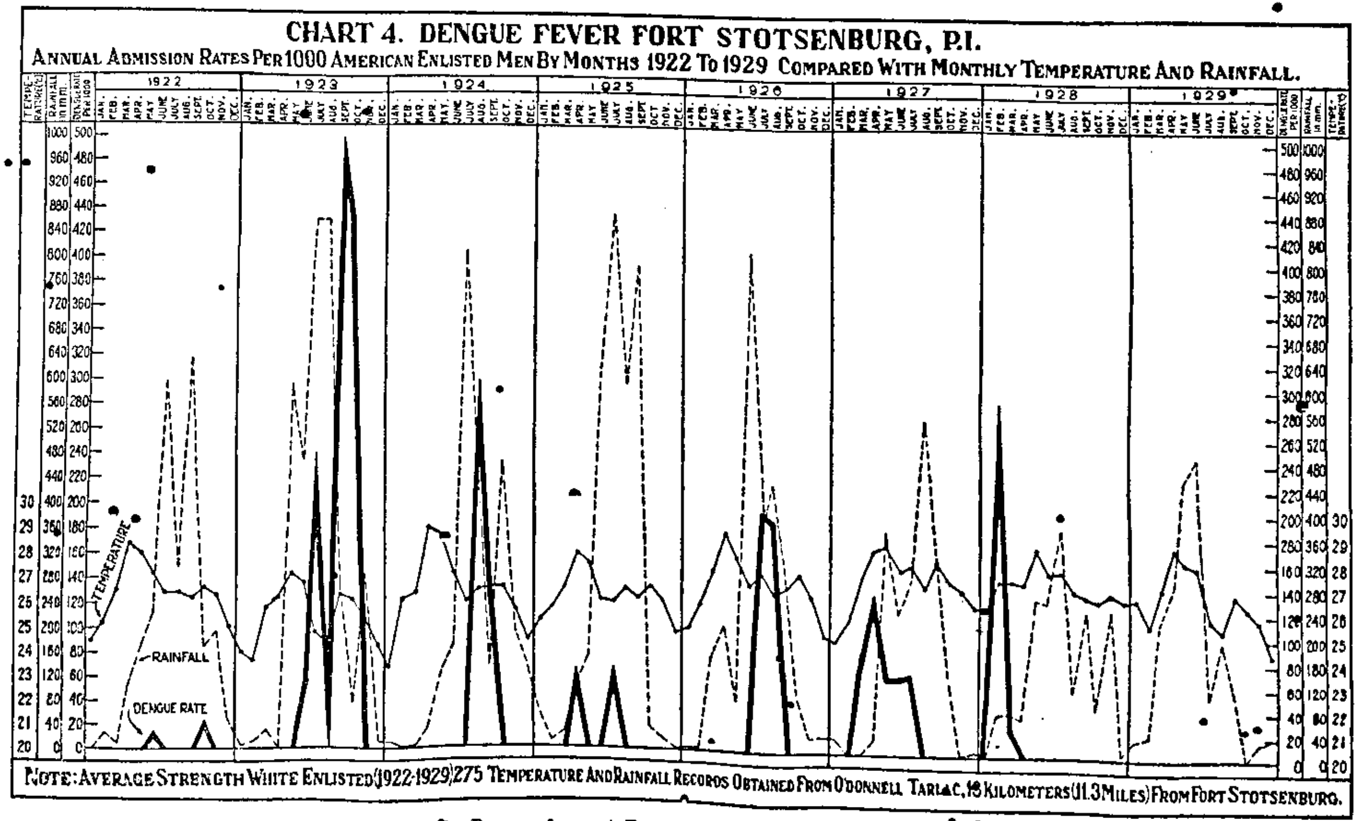

FIG. 4. Dengue fever at Fort Stotsenburg, Philippine Islands.

Fort Mills occupies a portion of a small island, Corregidor, situated in the mouth of Manila Bay about 48 kilometers from the City of Manila. The island rises abruptly from the sea to an elevation of about 500 feet. The temperature range for Corregidor during 1928 was as follows: Minimum, 19.5° C.; maximum, 36° C.; mean, 26° C.; and the total rainfall was 116.60 inches.

The dengue vectors, *Aëdes ægypti* and *Aëdes albopictus*, have been found breeding on the island, and were collected there by the authors during 1928. The white enlisted strength of this post was 2,352 in 1922, 1,057 in 1923, 1,363 in 1924, 1,406 in 1925, 1,372 in 1926, 1,264 in 1927, 1,464 in 1928, and 1,592 in 1929.

The incidence of dengue among troops at Fort Mills during the past seven years is indicated in fig. 5.

Nichols Field, which is located just outside Manila, has practically the same rainfall and temperature range as that of the city. The average strength for American troops was 296 in 1922, 398 in 1923, 447 in 1924, 472 in 1925, 473 in 1926, 454 in 1927, 448 in 1928, and 532 in 1929. As shown in fig. 6, dengue is not infrequent at this station.

Fort William McKinley, which is situated about 9 miles southeast of Manila at an elevation of not more than 125 feet, has practically the same average rainfall and temperature as Manila. While it is one of the largest stations in the Islands, the average strength for white American troops is relatively small as follows: 247 in 1922, 551 in 1923, 851 in 1924, 993 in 1925, 949 in 1926, 927 in 1927, 782 in 1928, and 409 in 1929. The dengue admission rates for this post are indicated in fig. 6.

Troops in the City of Manila.—The City of Manila is situated on Manila Bay in latitude 14° 35′ 31″ north and longitude 120° 58′ 08″ east, at sea level. During 1928 the total rainfall was 1,758.8 millimeters; and the maximum, mean, and minimum temperatures were, approximately, 36.7° C., 26.3° C., and 17.4° C., respectively. Troops stationed in the city include the personnel of the Sternberg General Hospital and the Post of Manila.

The dengue rates for all American troops stationed in Manila have been extremely high, as shown in figs. 3 and 6, in fact 69 per cent of all the dengue cases reported for the entire Army during 1928 occurred in this city. The importance of this source of dengue infection is further emphasized by the fact that if the adjacent stations, Fort William McKinley and Nichols Field, are

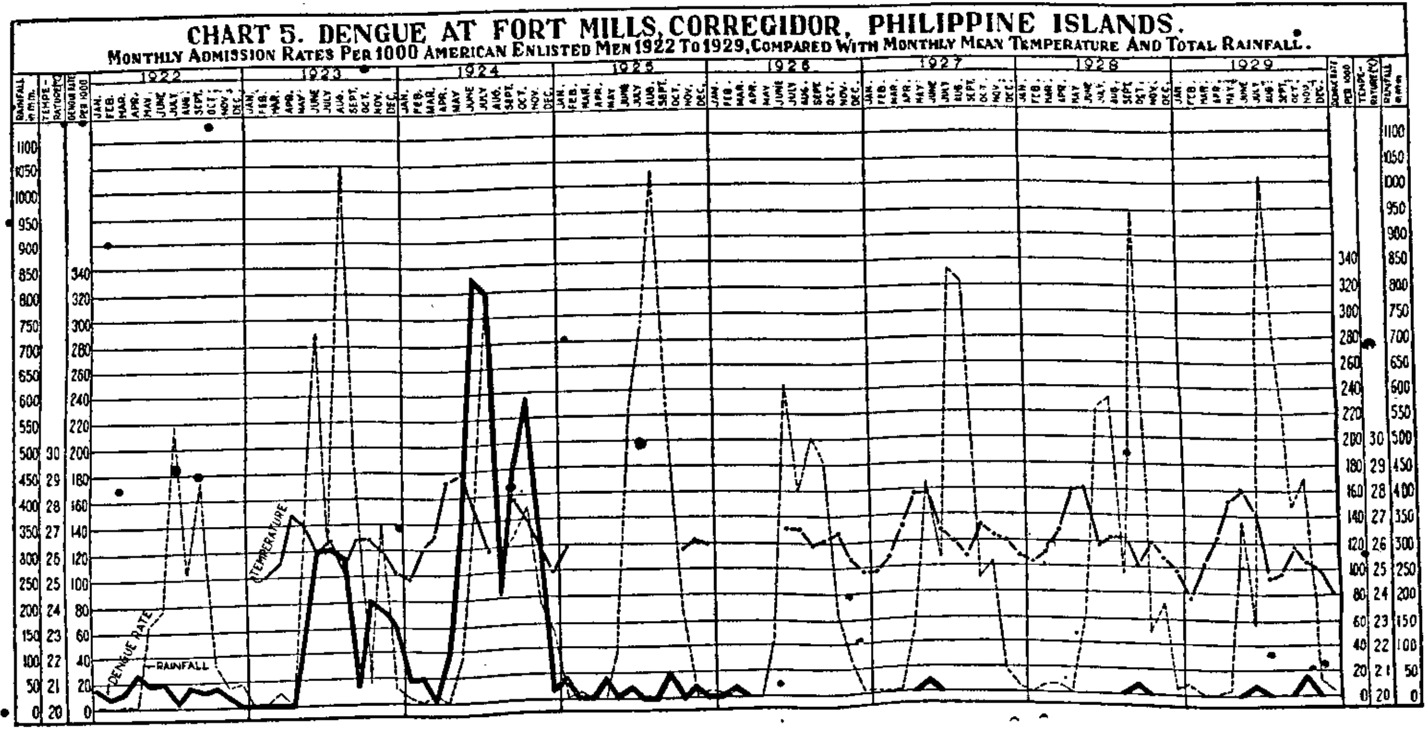

Fig. 5. Dengue at Fort Mills, Corregidor, Philippine Islands.

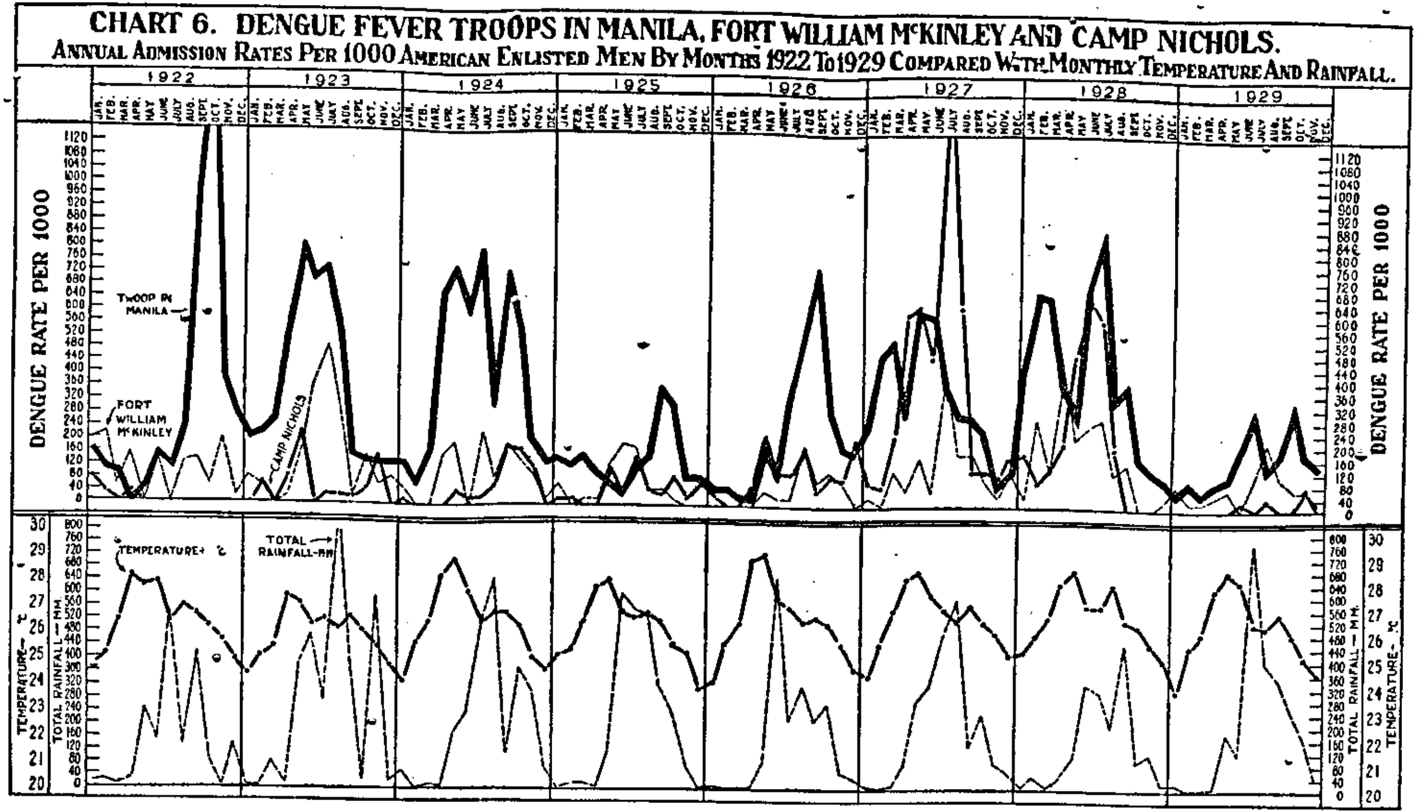

FIG. 6. Dengue fever among troops in Manila, at Fort William McKinley, and at Camp Nichols.

included, it may be stated that 98.2 per cent of the cases among troops in the Philippines, and 96.2 per cent of all cases reported among the 111,340 white troops in the entire United States Army during 1928 occurred among 2,406 American soldiers stationed in and near Manila.

DISCUSSION

From these observations it is apparent that the only important focus from which the American soldier is likely to contract dengue at present is in Manila or the immediately adjacent territory. The decided differences in the incidence of the disease among troops in the continental United States, Hawaii, Panama, and the Philippines cannot be explained entirely by climatic or other intrinsic differences in these various countries. While dengue may not be so common among the civil population of the United States or Hawaii as in the Philippines, still the disease is known to exist in both places, and epidemics have occurred in each of these countries as well as in Panama and in the Philippines. It is also known that an effective vector of dengue, *Aëdes ægypti,* is prevalent in each of these countries. Furthermore, Panama with an insignificant admission rate of 0.5 per thousand for 1928, and the Philippine Islands with an extremely high rate of 177.06, are both tropical countries. Thus, factors other than climate must be considered. While differences in the proportion of susceptible individuals from the dengue-free northern parts of the United States, assigned to duty in the different countries, and their length of stay no doubt influence the rates, it is believed that the most important factor is the degree of effectiveness with which troops are protected from dengue-infected mosquitoes in the various localities. In the Panama Canal Zone and surrounding areas the employment of every available scientific facility in mosquito prophylaxis has not only resulted in the disappearance of yellow fever and a marked decrease in malaria, but undoubtedly has also been responsible for the extremely low dengue rates among soldiers stationed there. Army posts both in the United States and Hawaii not only have the advantage of the mosquito control work of Army sanitarians, but also are to a large degree protected from dengue of civilian origin by the excellent sanitary conditions in neighboring towns and cities, where it is the custom for the people to live in screened houses. Screening alone undoubtedly plays an important part in reducing the proportion of mosquitoes infected and in keeping the dengue rates at a minimum in these localities. On the other hand, conditions in the Philippines are particularly favorable to the spread of dengue. Not only is the climate ideal for mosquito breeding, but also the lack of effective mosquito control among the civil population results in the development of enormous numbers of the vectors, *Aëdes ægypti* and *Aëdes albopictus;* while the custom, among both native and white civilians, of living in unscreened houses no doubt results in the exposure of a large proportion of dengue cases to innumerable mosquitoes. The latter factor alone would result in a much higher percentage of infected vectors than in the other countries mentioned. The introduction of new groups of nonimmune recruits into such highly infected areas of the Philippines at frequent intervals is inevitably followed by large numbers of dengue infections. However, in certain of the nine isolated Army posts in the Philippines active mosquito-control work and the screening of infected persons have resulted in a reduction of dengue rates. For instance, at Fort Stotsenburg which is removed from large towns or cities, the local mosquito-control work is also apparently effective in keeping down the rates for dengue. On the other hand, it is impossible by control work on the Army posts alone to protect troops stationed in Manila from dengue completely, so long as the soldiers are continually exposed to the great numbers of dengue-infected mosquitoes from other parts of this *Aëdes*-infested, unscreened city.

SEASONAL OCCURRENCE

Dengue occurs in seasonal waves in the Philippines, and as a rule these waves reach a high point some time between April and November. The monthly incidence of dengue in the Philippines between January, 1922, and December, 1929, is shown in fig. 7, along with the monthly temperature and rainfall for Manila. The mean monthly temperature curve for each year is lowest from November to April and reaches its height during the remaining months. The monthly rainfall, which varies from year to year, apparently plays an important part in determining the period of greatest mosquito prevalence, and consequently the time at which dengue is most prevalent. For example, by

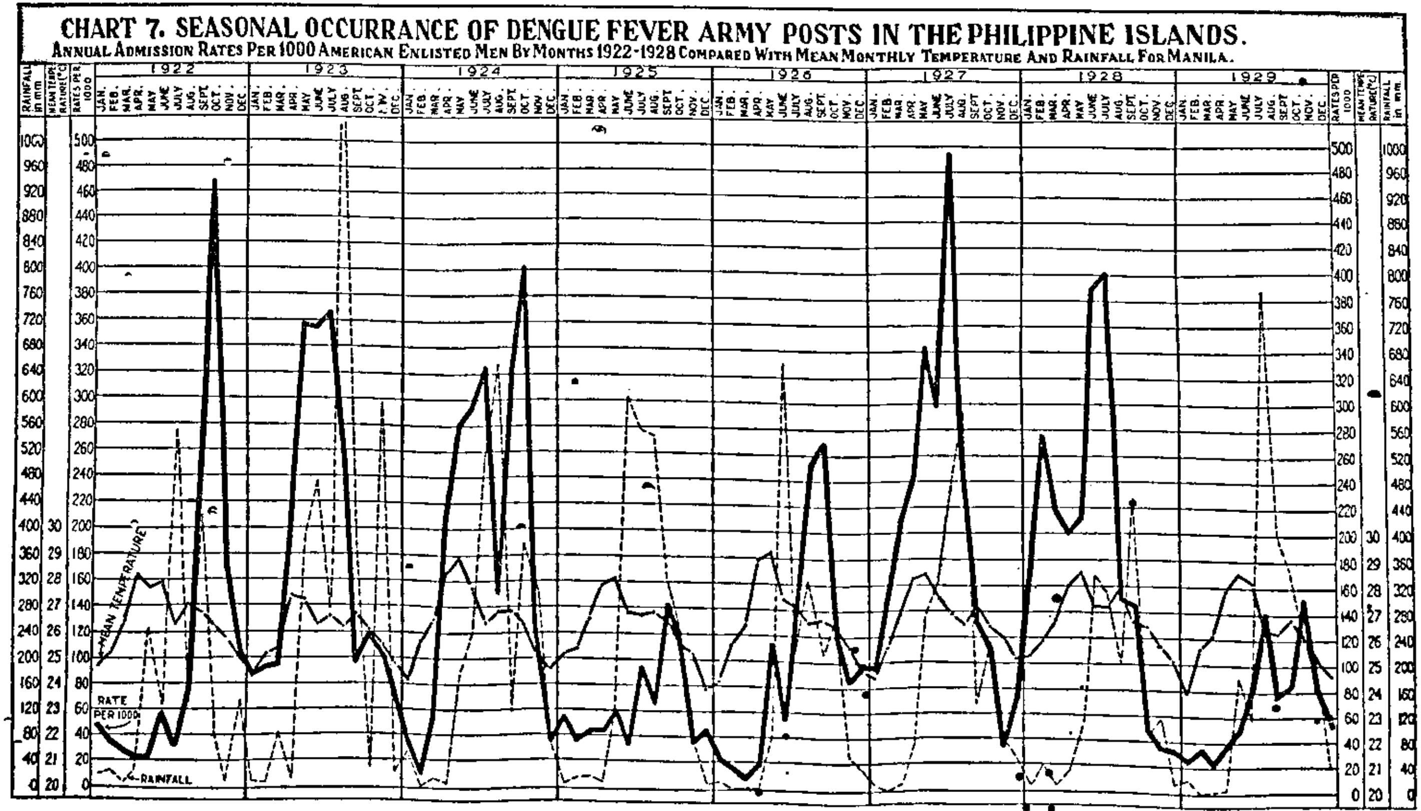

CHART 7. SEASONAL OCCURRANCE OF DENGUE FEVER ARMY POSTS IN THE PHILIPPINE ISLANDS.

ANNUAL ADMISSION RATES PER 1000 AMERICAN ENLISTED MEN BY MONTHS 1922-1928 COMPARED WITH MEAN MONTHLY TEMPERATURE AND RAINFALL FOR MANILA.

FIG. 7. Seasonal occurrence of dengue fever at Army posts in the Philippine Islands.

making daily observations throughout the period 1922 to 1924, Siler, Hall, and Hitchens determined that the late seasonal rise in dengue during 1922 was due to the peculiar daily distribution of rainfall during that year.

RACIAL DISTRIBUTION

It is unfortunate that more complete information is not available concerning the incidence of dengue among the native inhabitants of the Philippines. Such data would, no doubt, help to answer certain important questions concerning dengue immunity and might throw further light on the method of maintenance and dissemination of the virus in endemic localities. However, some information concerning dengue among adult male Filipinos is made available by the official records concerning Philippine Scout soldiers. As indicated in fig. 8 the annual dengue admission rates for Filipino troops since 1915 have been consistently much lower than the rates for American troops. It would appear that while dengue does occur among adult Filipinos, the native Scout soldiers as a group are not nearly so susceptible as American troops. This conclusion is substantiated by the fact that attempts to transmit dengue to Scout soldiers experimentally, both by Siler, Hall, and Hitchens and by the authors, have almost invariably failed. The former workers advanced the theory that the relative immunity of adult Filipinos is not a racial characteristic, but has probably been developed as a result of repeated exposure to infection since childhood. This supposition is concurred in, particularly since it has recently been proved that adult natives living in dengue-free mountain provinces of northern Luzon are just as susceptible to experimental infection with the disease as are white men.

Granting that native children are as a rule susceptible to dengue, it becomes apparent that the introduction of susceptible individuals from elsewhere is not necessary to the continued maintenance of the virus in an endemic area, but that the advent of children each year is sufficient to keep up the cycle of transfer indefinitely, even in isolated native communities. In addition, it is quite probable that mild unrecognizable infections may occur in many adults, as has been proved possible in monkeys, and that the virus can be transmitted from these apparently symptomless cases of dengue.

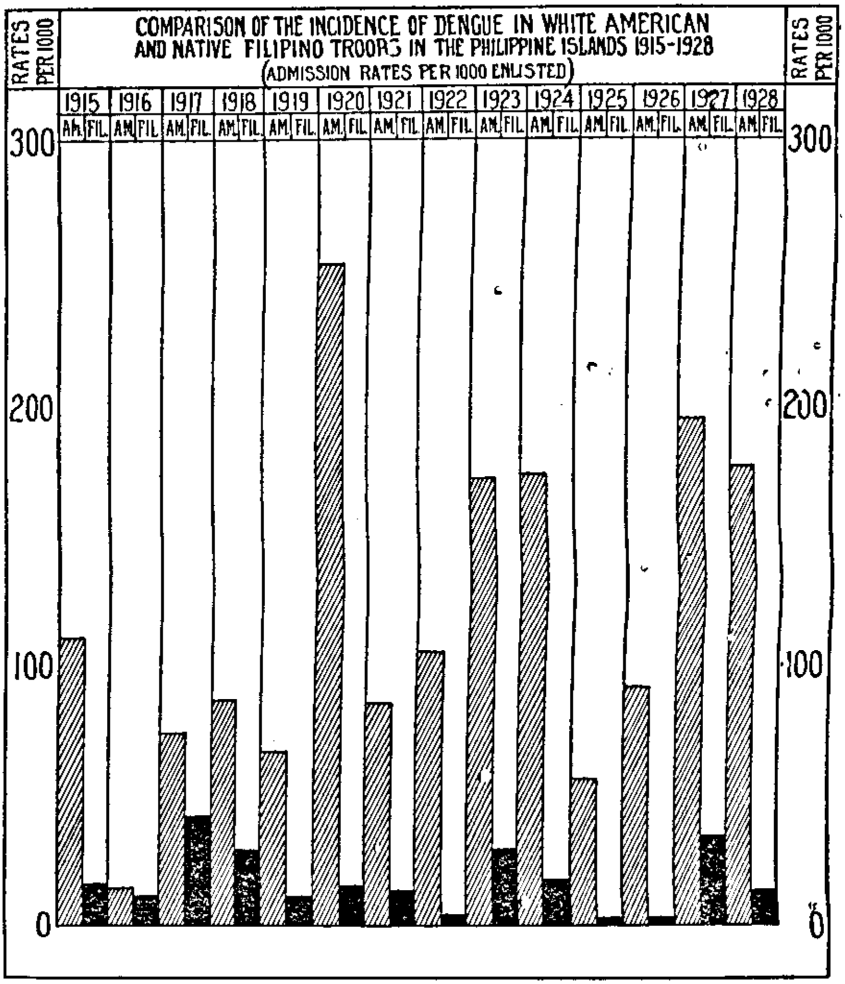

FIG. 8. Comparison of dengue incidence in white American and native Filipino troops in the Philippine Islands, 1915–1928.

ANIMAL AND INSECT RESERVOIRS OF DENGUE VIRUS

Another factor which demands consideration is the possibility of maintaining a reservoir of dengue virus by passage of the infection through animals, or by direct transfer of the virus from infected to normal mosquitoes. The authors have proved that at least a small proportion of adult monkeys living in endemic areas and a larger proportion from dengue-free localities may develop dengue without recognizable symptoms, and that from these monkeys the virus may be transmitted through mosquitoes to human beings. It has also been shown that under artificial conditions normal mosquitoes can be infected by the ingestion of suspensions of dengue-carrying mosquitoes, which suggests the possibility of such transfer of infection under natural conditions.

It, therefore, appears almost impossible to control the many unrecognizable sources of mosquito infection. While the protection of dengue-fever cases from mosquitoes by screening is a logical and advisable control measure, it seems quite probable that even if all recognized dengue cases were so protected, mosquito infection could not be prevented. Therefore, the most effective methods of control should be those that are directed mainly toward the reduction of the insect vectors.

INSECT VECTORS IN THE PHILIPPINES

At least two important vectors of dengue, *Aëdes ægypti* and *Aëdes albopictus*, are prevalent in the Philippine Islands. It has been proved that both of these mosquitoes are susceptible to infection with dengue virus and are effective in transmitting the virus to man. There is, of course, the possibility that other insects may also be concerned in the dissemination of the disease under natural conditions.

SUMMARY AND CONCLUSIONS

1. Dengue is of importance to the United States Army chiefly because of its high incidence among white American troops stationed in or near the mosquito-infested, endemic dengue area in and adjacent to the City of Manila, Philippine Islands.
2. Two important vectors, *Aëdes ægypti* and *Aëdes albopictus*, are known to be prevalent in the Philippines. It is of course possible that further investigation may show that other insects, including various species of mosquitoes and phlebotomus, are also capable of transmitting the disease.
3. Dengue shows a seasonal variation corresponding to changes in the numbers of mosquito vectors available, which factor is in turn influenced by variations in the rainfall and temperature.
4. In the Philippines the dengue admission rates for white American soldiers are much higher than for Philippine Scout soldiers.

5. The immunity of the adult Filipino soldier is probably due to repeated exposure to dengue during childhood.

6. The possibility is mentioned that dengue virus may be maintained in an endemic area by transfer through (*a*) susceptible native children, (*b*) incompletely immune native adults, (*c*) susceptible newcomers from dengue-free localities, (*d*) susceptible young animals or incompletely immune adult animals, and possibly (*e*) by direct transfer from infected to normal insect vectors.

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TRANSMISSION OF DENGUE BY AËDES ÆGYPTI

The great importance of *Aëdes ægypti* as a dengue vector was fully demonstrated by the experiments of Bancroft,(1) Cleland, Bradley, and McDonald,(2) and Siler, Hall, and Hitchens;(3) and information concerning the mechanism of its transmission of dengue was furnished by the extensive investigations of the last-mentioned group of workers. During the present studies, including a large number of experiments in which were employed one hundred thirty-one human volunteers, forty-three monkeys, many smaller laboratory animals, and two hundred twenty-six lots of mosquitoes, mostly *A. ægypti* (see fig. 9), considerable information has accumulated bearing on the mechanism of dengue transmission by this mosquito. These observations are of interest chiefly because they substantiate the conclusions of Siler, Hall, and Hitchens(3) concerning *A. ægypti* and prove that the findings of these authors may be safely applied to general experimental work with this mosquito. So far as possible throughout the present studies the normal mosquitoes were fed on infective blood during the first or the second day of dengue; and were used for the transmission of dengue only after twelve or more days had elapsed. Laboratory-bred *A. ægypti* descended from the stock used by Siler, Hall, and Hitchens in 1924 and a single strain of dengue virus were used in all of the experiments.

PERIOD DURING WHICH THE BLOOD OF DENGUE PATIENTS IS INFECTIVE TO AËDES ÆGYPTI

As the primary consideration in our work was to infect the mosquitoes, an effort was made in all cases to feed the normal *Aëdes ægypti* on dengue patients as early as possible after the onset of the disease. Of thirty-four lots of *A. ægypti,* Table 1, which took blood from cases of experimental dengue fever in which the onset was definitely known, twenty-nine ingested the infective blood during the first twenty-four hours, four in the second day, and one lot on the third day. The only failure occurred with *A. ægypti* of lot 11, which took blood from an American volunteer (case A.V. 5–M), who for three days previously

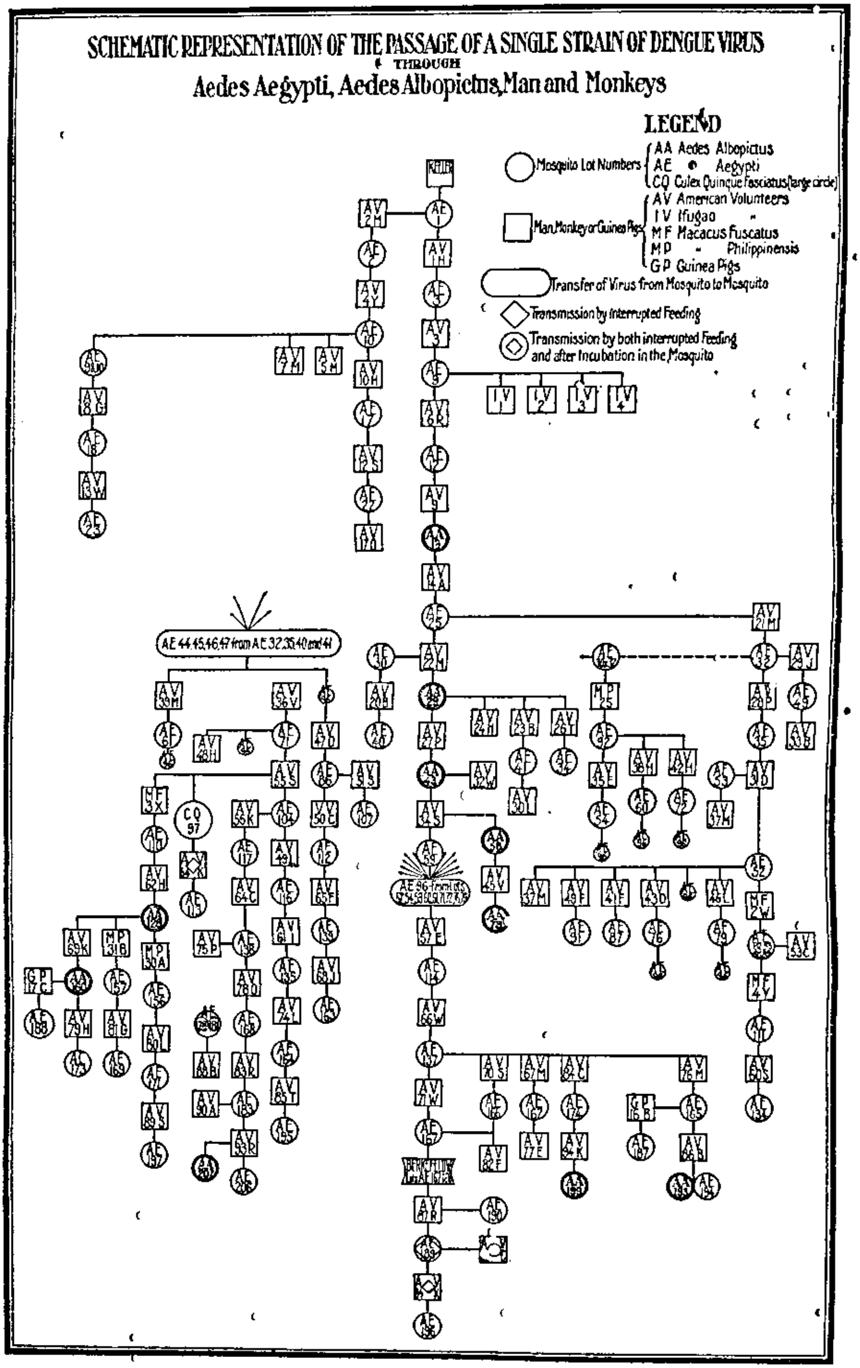
SCHEMATIC REPRESENTATION OF THE PASSAGE OF A SINGLE STRAIN OF DENGUE VIRUS
THROUGH
Aedes Aegypti, Aedes Albopictus, Man and Monkeys
LEGEND
Mosquito Lot Numbers
AA Aedes Albopictus
AE " Aegypti
CQ Culex Quinquefasciatus (large circle)
Man, Monkey or Guinea Pigs
AV American Volunteers
IV Ifugao "
MF Macacus Fuscatus
MP " Philippinensis
GP Guinea Pigs
Transfer of Virus from Mosquito to Mosquito
Transmission by interrupted Feeding
Transmission by both interrupted Feeding and after Incubation in the Mosquito
AE 44,45,46,47 from AE 32,35,40 and 41

FIG. 9. Schematic representation of the passage of a single strain of dengue virus through various hosts.

TABLE 1.—*Interval between the feeding to infect Aëdes ægypti and subsequent use in dengue-transmission experiments.*

Interval in days.	Lot Nos.[a]	Total volunteers used.	Dengue fever.	Interval in days.	Lot Nos.[a]	Total volunteers used.	[a] Dengue fever.
1	100	1	No.	27	112	1	Yes.
3	25	1	No.	29	173	1	Yes.
5	25, 69[b]	2	No.	30	71, 174	2	Yes.
7–9	178,[b] 181[b]	1	No.	31	32	1	Yes.
12	17, 32, 96	3	Yes.	32	52	3	Yes.
13	11	2	No.	37	19, 52, 171	3	Yes.
	30	1	Yes.	40	3, 10, 198	3	Yes.
14	53, 69,[b] 86, 116	4	Yes.	41	2, 52, 139	3	Yes.
15	25, 104, 162	5	Yes.	42	137	1	Yes.
15–17	178,[b] 181[b]	1	Yes.	45	9, 135, 195	3	Yes.
16	11	1	No.	46	165	1	Yes.
	1, 18	3	Yes.	47	137	1	Yes.
17	117	1	Yes.	48	138	1	Yes.
18	49	1	Yes.	52	10, 137, 164	4	Yes.
19	167, 168	2	Yes.	54	190	1	Yes.
20	41	1	Yes.	55	9	3	Yes.
22	12, 85	2	Yes.	57	138	1	Yes.
24	114	1	Yes.	57–59	9 and 10 combined	1	Yes.
25	71, 86	2	Yes.	60	9	1	Yes.
25–29	167, 168	1	Yes.	70	137	1	Yes.

[a] All lots except lot 11 were proved infected in twelve or more days following the infective feeding.

[b] Lots 69, 96, 178, and 181 were infected by feeding on suspensions of dengue-infected mosquitoes, while all other lots were infected by feeding on experimental cases of dengue fever within forty-eight hours after the onset, excepting lot 11; this lot was fed during the third day after the first appearance of symptoms.

had shown marked prodromal symptoms. While the day of onset could not be definitely determined in this case, it is believed that the mosquitoes were fed on the third day, and that this accounted for the fact that they remained uninfected.

While exact experimental information is not available to show that each individual mosquito in the lots fed during the first two days of the dengue was infected, the fact that the disease was transmitted by all of these lots regardless of the numbers of mosquitoes used indicates that the infective rate must have been high.

TIME REQUIRED BEFORE AËDES ÆGYPTI, FED ON MATERIAL CONTAINING DENGUE VIRUS, WERE ABLE TO TRANSMIT THE DISEASE

It was concluded by Siler, Hall, and Hitchens (3) that the virus of dengue "must remain in the female *Aëdes* for a period of more than ten days—and that the evidence indicates that even on the eleventh day after infection of *Aëdes* the virus may not have reached such a state that the bite of the mosquito is constantly infective." While successful dengue transmission has occasionally been obtained following a shorter period of virus "development," as, for example, in the experiment reported by Schule(4) in which the period was only eight days, an interval of at least eleven days was usually allowed in the present work. Exceptions to this rule were a few lots used as controls in other experiments. For example, lot 25 fed on the third and fifth days, lot 69 fed on the fifth day, lot 178 fed on the ninth day, and lot 181 fed on the seventh day after the ingestion of dengue virus failed to transmit the infection to susceptible volunteers (Table 1). However, all of these lots were proved to be infective when used after longer periods of time had elapsed. In sixty-three successful transmission experiments with *A. ægypti* the intervals between the ingestion of infective blood and the subsequent test of the mosquitoes for infectivity were from twelve to seventy days (Table 1). Three of these lots (17, 32, and 96) were tested on the twelfth day after the meal of infective blood.

DURATION OF THE PERIOD OF INFECTIVITY IN AËDES ÆGYPTI

Siler, Hall, and Hitchens,(3) who reported the transmission of dengue by mosquitoes that had been infected sixty-two, sixty-six, and seventy-five days previously, made the following statement: "We believe the evidence justifies the statement that after the female *A. ægypti* has become infective she is capable of transmitting the virus of dengue throughout the remainder of her natural life." Blanc and Caminopetros(5) have been able to keep *A. ægypti* alive for seven months and have transmitted dengue with them one hundred seventy-four days after infection. Their observations concerning the influence of low temperatures on the period of infectivity are of particular interest as they may answer some of the obscure questions concerning the epidemiology of the disease. According to these workers infective *A. ægypti* may lose their ability to transmit dengue virus if kept at temperatures below 18° C.; and they state that mosquitoes kept in Athens remained infective up to January 13, but that the same mosquitoes were not infective during the cold months of February and March. In this connection it is interesting to note the record of our lot 190. Immediately after an infective blood feeding a portion of this lot was kept in the refrigerator at 18° C. for thirty days and subsequently at room temperature (26.3° C.) for twenty-four days, after which the mosquitoes were able to transmit dengue.

In the present work all except a portion of one lot were used in Manila where the maximum, mean, and minimum temperatures for the period were 36.7° C., 26.3° C., and 17.4° C., respectively. The lot that was used outside of Manila was taken to Baguio, Mountain Province, in April, 1929, and used the following day in transmitting dengue to each of four Igorot volunteers.

The longest periods of infectivity observed in the various lots happened to be sixty days with lot 9, and seventy days with lot 137 (see Table 1). Either because of death of the mosquitoes or for other reasons no lots were tested after longer periods of time.

Thus it may be stated that in the present work in Manila *A. ægypti* fed on infective dengue virus were subsequently able, in all instances, to transmit dengue between the twelfth and seventieth days.

NUMBERS OF INDIVIDUAL MOSQUITOES USED IN THE TRANSMISSION OF DENGUE

The numbers of potentially infected *Aëdes ægypti* used in successful transmission experiments varied from five to one hundred fifty mosquitoes for each volunteer. As shown in Table 2, over 8 per cent of the infections followed the feeding of from one hundred to one hundred fifty mosquitoes; 21.66 per cent were caused by fifty to one hundred mosquitoes; 43.33 per cent by ten to fifty mosquitoes; and 26.66 per cent of the cases were due to the bites of from five to ten mosquitoes. As no negative results were obtained except in the case of individuals later proved to be immune, it is apparent that small numbers of infective mosquitoes may be used for dengue transmission with the same certainty of positive results as if larger groups were employed. As sixteen, or 26 per cent, of the cases were infected by different lots of *A. ægypti,* all of which were made up of between five and ten individual mosquitoes, and as there were no failures after using such small numbers, it is apparent that at least one out of ten mosquitoes in these particular lots were infective. However, it is believed that such an estimate is far from exact; and since infections have been produced with one, two, or three individual *A. albopictus,* (6) it seems quite probable that of all normal *A. ægypti* which take blood from dengue cases during the infective period practically 100 per cent become infective, and that any individual mosquito in the group may become able to transmit dengue.

TABLE 2.—*Numbers of Aëdes ægypti used in the transmission of dengue to man and monkeys.*

Infective *A. aegypti:* lot Nos.	Number of mosquitoes fed.	Volunteers or monkeys infected with dengue.		Per cent of total cases.
		Number of cases.	Per cent.	
9	5	1	100	26.66
9, 52, 137	6	4	100	
9, 137	7	2	100	
32, 52, 71 (9 and 10)	8	4	100	
30, 52	9	2	100	
52, 86, 112	10	3	100	
Total for group	5– 10	16	100	
1, 12, 17, 19, 25, 41, 86, 100, 168, 171	10– 20	12	100	43.33
104, 137, 138, 165 (44, 45, 46, and 47) (9 and 10)	20– 30	7	100	
96, 100	30– 40	2	100	
9, 53, 135 (44, 45, 46, and 47) (178 and 181)	40– 50	5	100	
Total for group	10– 50	26	100	
2, 3, 49, 117, 69	50– 60	5	100	21.66
71, 137, 138	60– 70	3	100	
189	70– 80	1	100	
18, 35, 137, 162	80– 90	4	100	
Total for group	50–100	13	100	
10, 114, 139, 167	100–150	5	100	8.33
Total infections	5–150	60	100	100.00

SUMMARY AND CONCLUSIONS

1. The results of these experiments indicate that all lots of *Aëdes ægypti* that fed on blood from experimental cases of dengue during the first forty-eight hours of the disease became infected; while one lot which probably took blood during the third day of the disease failed to transmit the disease.

2. In a few experiments in which *A. ægypti*, one, three, five, seven, and nine days after they had ingested virus, were allowed to feed on volunteers the mosquitoes failed to transmit the infection to susceptible volunteers. However, all transmission experiments in which the intervals were from twelve to seventy days were successful.

3. No evidence was obtained to indicate that the infectivity of the mosquitoes was ever lost at the temperatures that prevail in Manila. Lots infected sixty and seventy days previously transmitted dengue as did mosquitoes infected for shorter periods.

4. It appears that when a group of normal *A. ægypti* ingests dengue virus a large proportion of the individual mosquitoes become infected. Dengue was transmitted by small lots composed of five to ten mosquitoes just as consistently as by large lots made up of one hundred to one hundred fifty mosquitoes.

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TRANSMISSION OF DENGUE BY AËDES ALBOPICTUS SKUSE

While *Aëdes ægypti* is the only species that has been generally accepted as a vector of dengue during the past few years, several other mosquitoes, including species of *Aëdes* and *Culex*, have been suspected either on epidemiological or experimental grounds. The recent discovery by Bauer (1) that several species of *Aëdes* are capable of transmitting yellow fever suggested the advisability of investigating the prevalence in the Philippines of species of *Aëdes*, other than *A. ægypti*, in order to determine the possibility of their acting as dengue vectors.

Collections of larvæ and adults from Manila and adjacent localities have yielded only two species of *Aëdes;* namely, *Aëdes ægypti* and *A. albopictus*. Attention of the board was directed to *A. albopictus* in February, 1929, during the examination of the first group of adult mosquitoes submitted for examination, from Corregidor Island. The results obtained in collections of larvæ made in representative parts of Manila during April, May, June, July, and August, 1929, indicated, as shown in Table 3, that both *A. ægypti* and *A. albopictus* are common mosquitoes in this locality. Hence, it was concluded that if *A. albopictus* were able to transmit dengue, its importance might be about the same as that of *A. ægypti*.

TABLE 3.—*Relative prevalence of Aëdes albopictus Skuse and A. ægypti in Manila, as indicated by collections of larvæ between April and September, 1929.*

Month.	Number of samples collected.	Samples containing *A. albopictus.*	Predominant species in samples.	Dengue cases admitted to Sternberg General Hospital, Manila.
		Per cent.		
April	1	100	*A. ægypti*	18
May	8	100	do	16
June	4	75	*A. albopictus*	38
July	16	100	Neither	33
August	104	43	*A. ægypti*	11

While systematic collections of *Aëdes* larvæ have not been made since August, 1929, adult *A. albopictus* have been identified in various localities. Specimens have been caught at Fort William McKinley by Maj. John Dibble, M. C., United States Army; it has been found as commonly as *A. ægypti* around Novaliches, Rizal Province, Luzon, by Dr. C. Manalang, chief of the malaria-control division of the Philippine Health Service; and specimens have been taken in Manila by Dr. Paul F. Russel, field director, International Health Division, Rockefeller Foundation. *Aëdes albopictus* has also been seen in the City of Manila during every month of the year by the authors, and during February and March, 1930, this mosquito was found to be the predominant species around the Manila residence of one of the authors.

Not only is *A. albopictus* a common local mosquito at present, but there is sufficient evidence to indicate that it has been prevalent in the Philippine Islands during the past. For example, Ludlow (2) in 1903 described this mosquito under the name of *Stegomyia scutellaris samarensis;* and in 1908 (3) remarked that "in places where *Stegomyia calopis* was infrequent its place was largely taken by *Stegomyia scutellaris.*" Banks (4) in 1906 reported that he had failed to collect *S. scutellaris* Walker, but that *S. scutellaris samarensis* Ludlow "seemed a widespread mosquito in the Philippines." He noted that *S. scutellaris* had been found in the following locations: Pangasinan, Camp Gregg, Bayambang (Col. W. P. Chamberlain, M. C., U. S. Army), Samar, Leyte, Mindoro, Iloilo, Occidental Negros, Bago (Banks), Manila, Fort William McKinley (Col. Charles F. Craig, M. C., U. S. Army). As the names *S. scutellaris* Walker and *S. scutellaris samarensis* Ludlow are accepted synonyms for *A. albopictus* Skuse, it is apparent, from the information presented, that this common Oriental mosquito is widely distributed throughout the Philippine Archipelago. That *A. albopictus* is also prevalent throughout the Orient is indicated by the following observation made by Theobald (5) in 1901 concerning *Stegomyia scutellaris* Walker (*A. albopictus*):

> *Habitat.*—Singapore (Rafflesian Museum) (4. 9. 1899); Hong Kong (Ford) (27. 9. 1899); Selangor (A. L. Butler) (28. 10. 1899); Upper Burma (Watson); North Borneo; Mauritius (Sir Charles Bruce) (22. 11. 1899); Tamsui, Formosa (Mackay) (2. 8. 1899); Fiji (Black) (30. 12. 1899); Japan (Wood); Celebes (Walker); Ceylon (Bartholomew) (12. 12. 1899); Madras and Naini Tal, India (Giles and Cornwall); Siam

> (Skeate); Amboina (Doleschall); Sombalpur, C. P., India (D. O'C. Murphy) (99); Foo-Chow, China (Rennie) (9. 8. 1900) (84).
>
> *Time of capture.*—Singapore, July (July 27, 1899); Ceylon, November; Upper Burma (March).
>
> *Observations.*—This is a very common mosquito, with a wide distribution in Asia. It is a common species in the Straits Settlements, being the second commonest mosquito in Selangor (A. L. Butler). Ford records its larvae as being abundant "in standing water near houses 500 feet above the sea." It is a great nuisance at Calcutta (Skuse). Skeate also evidently took it in abundance in Siam, for numbers are in the collection sent me by Dr. Sharpe.

Barraud,(6) in 1922, also called attention to the prevalence of *A. albopictus* and noted that there were specimens in the central Malaria Bureau collection from the following places:

> North-West Frontier:—Kohat (Sinton).
>
> Punjab:—Simla and Delhi (Christophers). Kasuali (Christophers and Sinton). Amritsar (Barraud).
>
> Bombay:—Islands in the harbour, and Trombay (Barraud).
>
> Bombay Deccan:—Belgaum and Nagargali (Barraud).
>
> Malabar Coast and Nilgiri Hills (Khazan Chaud).
>
> Madras Carnatic:—Madras (Patton). Salem (Christophers).
>
> Bihar; Cuttack (S. Sundar Rao). Burma:—Rangoon (Christophers).
>
> The following additional localities are taken from Theobald (Mon. Cul.):
>
> Calcutta (Annandale); Upper Burma (Watson); Madras and Naini Tal (Giles and Cornwall); Sombalpur, Cen. Prov. (D. O'C. Murphy); Kanara district (E. H. Aitken); Sylhet, Assam (Major Hall); Lushai Hills, Assam (E. C. Macleod); Manipur (C. A. Gourlay); Katihar and Purneah district; N. Bengal (C. A. Paiva); Ceylon (Green).

It is of interest to note that *Stegomyia scutellaris* (*A. albopictus*) has been previously suspected as a possible vector of dengue on epidemiological grounds; and that during the 1915 dengue epidemic in Formosa, it was used in dengue-transmission experiments by Koizumi, Yamaguchi, and Tonomura.(7) However, as was pointed out by these workers, their results were inconclusive because the human volunteers used had not been previously protected from accidental infections, and while the mosquito was incriminated, it was not proved to be capable of transmitting dengue.

EXPERIMENTAL

MATERIALS AND METHODS

(*a*) *Source of dengue virus.*—The strain of virus used in these experiments was obtained originally December 15, 1928, from a naturally acquired case of dengue (Keller) and had been passed alternately through various lots of mosquitoes and either man or monkey. As indicated in

Chart 1, the virus transferred from A.V.[2] 22–M to *A. albopictus* lot 29 and on to lot 43 and lot 58 had already passed through six different lots of *A. ægypti* and six white American volunteers. As shown in Chart 2, the virus used in infecting *A. albopictus* lots 128 and 160 had already passed through ten lots of *A. ægypti*, nine American volunteers, and one Japanese monkey, *Macacus fuscatus* 3 (X). The virus used in infecting *A. albopictus* lot 183 had passed through thirteen lots of *A. ægypti*, and twelve American volunteers as indicated in Chart 3.

(*b*) *Source of Aëdes albopictus.*—The mosquitoes selected for use in the first five transmission experiments and known as lot 29 had emerged both from larvæ collected in Manila and from others bred in the laboratory. All other lots (43, 58, 128, 160, and 183) were descended from laboratory-bred mosquitoes. The specific identity of each mosquito used, either for breeding or in the transmission experiments, was verified immediately after emergence, before and after being placed in the breeding cages, before and after taking an infective blood feeding from a dengue patient, and before and after the tests on volunteers. Each mosquito used conformed to the description of *A. albopictus* given by Theobald (5) and Barraud, (6) (see Appendix), and the identity of the species was further confirmed by the Bureau of Entomology, Washington, D. C.

(*c*) *Human volunteers and monkeys used in the transmission experiments.*—The twelve white American soldier volunteers[3] used in these experiments were selected and handled as in other transmission experiments carried out during this investigation. After admission to the special dengue ward at Sternberg General Hospital, Manila, they were isolated in screened cubicles within the screened ward, for eight or nine days before the beginning of the tests; or, if volunteers who were already in the ward were used, this period of time was allowed to elapse between previous negative experiments, and the date *A. albopictus* was tested for infectivity.

The two Philippine monkeys (*Macacus philippinensis*) used with *A. albopictus* were caught in a dengue-free region at an elevation of about 4,000 feet in the mountains of northern Luzon, transported in screened boxes to Manila, and kept in a screened cage until discarded. They had not been used previously for experimental work and had probably never been bitten previously by dengue-infected *Aëdes* mosquitoes. These animals were observed in screened cages for periods of one or more weeks before infected *A. albopictus* were allowed to feed on them.

Technic.—In the transmission experiments with *A. albopictus* the procedure was in general similar to that used in the work with *A. ægypti*. The female mosquitoes were identified, placed in a "feeding cage," and given an opportunity to take blood from an experimental case of dengue during the first or second day of the disease. After the mosquitoes had fed, they were held for twelve or more days before they were tested for infectivity. After the potentially infective biting, the volunteer or animal was examined daily for evidence of infection; and in all instances records were made of the symptoms, temperature, and leukocyte counts.

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[2] American volunteer.

[3] Hereafter referred to specifically as "A.V." with a number and a letter.

Dengue was recognized either by the appearance of typical symptoms including a definite fever with leukopenia and an increase in the immature granulocytes, or by passage of the virus through other mosquitoes, and again to man, causing subsequent infection. As shown in the following experiments, the six different lots of *A. albopictus* used produced dengue in two monkeys (*Macacus philippinensis*) and in twelve volunteers.

Experiments with Aëdes albopictus, lot 29.—July 16, 1929. One hundred recently emerged female mosquitoes were given an opportunity to feed on the infective blood of an experimental case of dengue (A.V. 22–M) during the first twelve hours of the disease. Eighty of these mosquitoes took full feedings of blood within a short time, were designated lot 29, and were used in the following experiments.

Experiment 1.—July 29, 1929. Twelve *A. albopictus* of lot 29, fed on a volunteer (A.V. 23–B) who had been used previously for negative experiments July 1, 11, and 20, and had been observed for nine days prior to this test. After an incubation period of seven days dengue fever developed on the morning of August 5, 1929. The symptoms were typical, including fever, both initial and terminal rash, weakness, pains and aches in the eyes, knees, and elbows, and leukopenia. The diagnosis was further confirmed by the fact that normal *A. ægypti* fed during the first day of the disease and, designated lot 41, later transmitted the disease to another volunteer (A.V. 30–L). Comment: The feeding of twelve infected *A. albopictus* of lot 29 on a human volunteer was followed in seven days by typical dengue fever.

Experiment 2.—July 29, 1929. Eight *A. albopictus* of lot 29 fed on a volunteer (A.V. 26–T), who had previously been used for negative experiments July 11 and 21, and had been observed for eight days, prior to this experiment. After an incubation period of six days, dengue fever developed in the afternoon of August 4, 1929. The symptoms, which were typical of dengue, included fever, generalized rash, and leukopenia. Comment: The feeding of eight *A. albopictus* of lot 29 on this volunteer was followed in six days by typical dengue fever.

Experiment 3.—August 6, 1929. Three *A. albopictus* of lot 29 fed on a volunteer (A.V. 25–D) who had been used previously for negative experiments July 11, 21, and 29, 1929, and had been observed for eight days prior to this test. After an incubation period of six and one-fourth days dengue fever developed during the afternoon of August 12, 1929, with the typical symptoms of a mild case, without any initial rash. The infection lasted five days, ending with a terminal rash August 17, 1929. Comment: The feeding of three *A. albopictus*, lot 29, was followed in six and one-fourth days with typical dengue fever.

Experiment 4.—August 6, 1929. One infected *A. albopictus* of lot 29 fed on a volunteer (A.V. 27–P) who had been used previously for negative experiments July 11, 21, and 29, 1929, and had been observed for eight days prior to this test. After an incubation period of five days dengue fever developed in the afternoon of August 11, 1929, with fever, anorexia, vomiting, dizziness, headache, pains in the muscles and eyes, and typical leukopenia. On the second day a rash appeared; general glandular enlargement was noted later in the disease. The diagnosis was further confirmed by the fact that normal *A. albopictus* fed during the first day of the disease, and designated lot 43, later transmitted dengue to other volunteers (A.V. 32–W and A.V. 34–S.). Comment: The feeding of one *A. albopictus*, lot 29, was followed in five days by typical dengue fever.

Experiment 5.—August 7, 1929. Ten *A. albopictus* of lot 29 fed on a volunteer (A.V. 24–H) who had previously been used in negative experiments July 11, 21, and 29, and had been observed for nine days prior to this test. After an incubation period of five and one-half days dengue fever developed August 12, 1929. The attack began abruptly with a chill, followed by fever, headache, generalized pains, and a flushing of the face. The typical symptoms, including pains and aches in the eyes and back, accompanied by leukopenia, persisted until August 15, 1929. Comment: The feeding of ten infected *A. albopictus*, lot 29, on this volunteer was followed after five days by typical dengue fever.

EXPERIMENTS WITH AËDES ALBOPICTUS, LOT 43

Fifty-four female normal *Aëdes albopictus*, which were descendants of the original stock bred in the laboratory, were infected August 12, 1929, by feeding on the blood of an experimental case of dengue (A.V. 27–P), during the first twelve hours of the disease. As this case of dengue had resulted from the bite of one mosquito of lot 29, the potentially infected group of lot 43 mosquitoes represented the transfer of the virus through a second lot of *A. albopictus*. The results are given in the records of experiments 6 and 7.

Experiment 6.—August 26, 1929. Twenty-two *A. albopictus* of lot 43 fed on a volunteer (A.V. 32–W) who had been used previously for a negative experiment August 17, 1929, and had been observed for nine days prior to this test. After an incubation period of five days, dengue fever developed August 31, 1929, beginning with headache, nausea, fever, and leukopenia. The disease, which lasted five days, was also characterized by generalized pains, nausea, vomiting, and dizziness. There was no rash. Comment: The feeding of twenty-two infected *A. albopictus* of lot 43 on this volunteer was followed after five days by typical dengue fever.

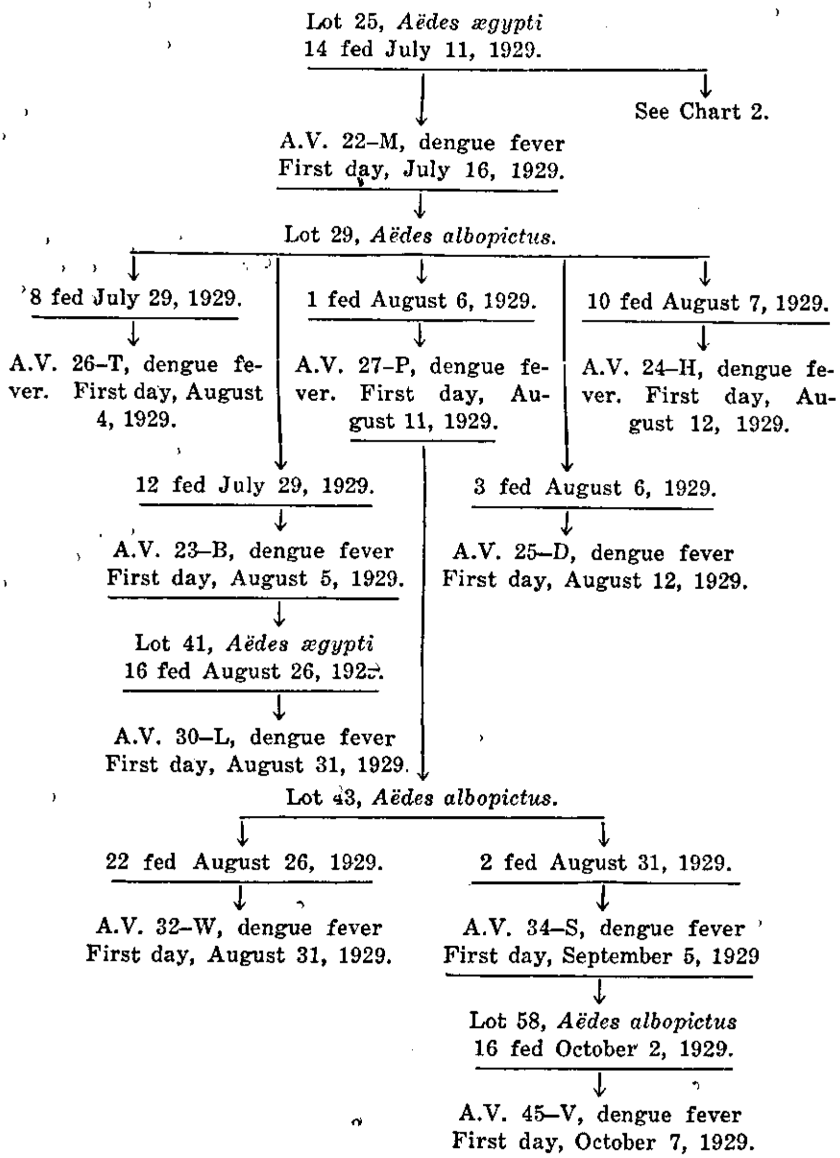
Experiment 7.—August 31, 1929. Two *A. albopictus* of lot 43 fed on a volunteer (A.V. 34–S) who had been admitted for this experiment alone, and had been observed for ten days prior to the test. After an incubation period of five days, typical dengue fever began September 5, 1929. The infection was characterized by headache, pains and aches in the eyes and muscles, weakness, dizziness, generalized rash, fever, and leukopenia. The duration of the disease was four and one-half days. Comment: The feeding of two infected *A. albopictus* of lot 43 on this volunteer was followed in eight days by typical dengue fever, proving that this mosquito is an effective dengue vector.

Experiment 8.—Sixteen normal *A. albopictus* were infected by feeding on the blood of American volunteer 34–S, September 6, 1929, and designated lot 58. October 2, 1929, three of these mosquitoes fed on A.V. 45–V and dengue fever developed October 7. The symptoms, which lasted for approximately four days, were typical, including a rash and leukopenia. Comment: Dengue fever developed in a susceptible individual following the feeding of three *A. albopictus* of lot 58.

CHART 1.—*Transfer of "K" strain of dengue virus from December 15, 1928, to July 16, 1929, by Aëdes ægypti and after that date by Aëdes albopictus, lots 29, 43, and 58.*

Keller, dengue fever
Second day, December 15, 1928.
↓
Lot 1, *Aëdes ægypti*
17 fed December 31, 1928.
↓
A.V. 1–H, dengue fever
First day, January 10, 1929.
↓
Lot 3, *Aëdes ægypti*
56 fed February 19, 1929.
↓
A.V. 3–A, dengue fever
First day, February 25, 1929.
↓
Lot 9, *Aëdes ægypti*
49 fed April 11, 1929.
↓
A.V. 6–R, dengue fever
First day, April 16, 1929.
↓
Lot 12, *Aëdes ægypti*
15 fed May 8, 1929.
↓
A.V. 9–D, dengue fever
First day, May 14, 1929.
↓
Lot 19, *Aëdes ægypti*
12 fed June 20, 1929.
↓
A.V. 14–A, dengue fever
First day, June 26, 1929.
↓
Lot 25, *Aëdes ægypti.*

CHART 1.—*Transfer of "K" strain of dengue virus from December 15, 1928, to July 16, 1929, by Aëdes ægypti and after that date by Aëdes albopictus, lots 29, 43, and 58*—Continued.



EXPERIMENTS WITH AËDES ALBOPICTUS, LOT 128

December 4, 1929, one hundred eighty-four normal female *A. albopictus* took infective blood from an experimental case of dengue (A.V. 62–H) during the second day of the disease. It is of interest to note here that the infection had been transferred to this volunteer through *A. ægypti*, lots 110–A, B, C, D, E, and F, from Japanese monkey (*Macacus fuscatus*) 2 (W).

CHART 2.—*Continuation of the passage of "K" strain of dengue virus through Aëdes ægypti from July 11, 1929, to November 28, 1929, and after that date by Aëdes albopictus of lots 128 and 160.*

Lot 25, *Aëdes ægypti*
15 fed July 11, 1929.

↓

A.V. 21–M, dengue fever
First day, July 17, 1929.

↓

Lot 32, *Aëdes ægypti*, combined with *A. ægypti* lots 35, 40, and 41
Fed to normal *A. ægypti* July 20, 1929.

↓

Lots 44, 45, 46, and 47 *Aëdes ægypti*
28 fed September 21, 1929.

↓

A.V. 36–V, dengue fever
First day, September 26, 1929.

↓

Lot 71, *Aëdes ægypti*
8 fed October 26, 1929.

↓

A.V. 55–S, dengue fever
First day, November 1, 1929.

↓ ↓

See Chart 3.

Blood inoculated.

↓

Macacus fuscatus 3 (X), dengue fever
between November 5 and 9, 1929.

↓

Lots 110–A, B, C, D, and E *Aëdes ægypti*
75 fed November 28, 1929.

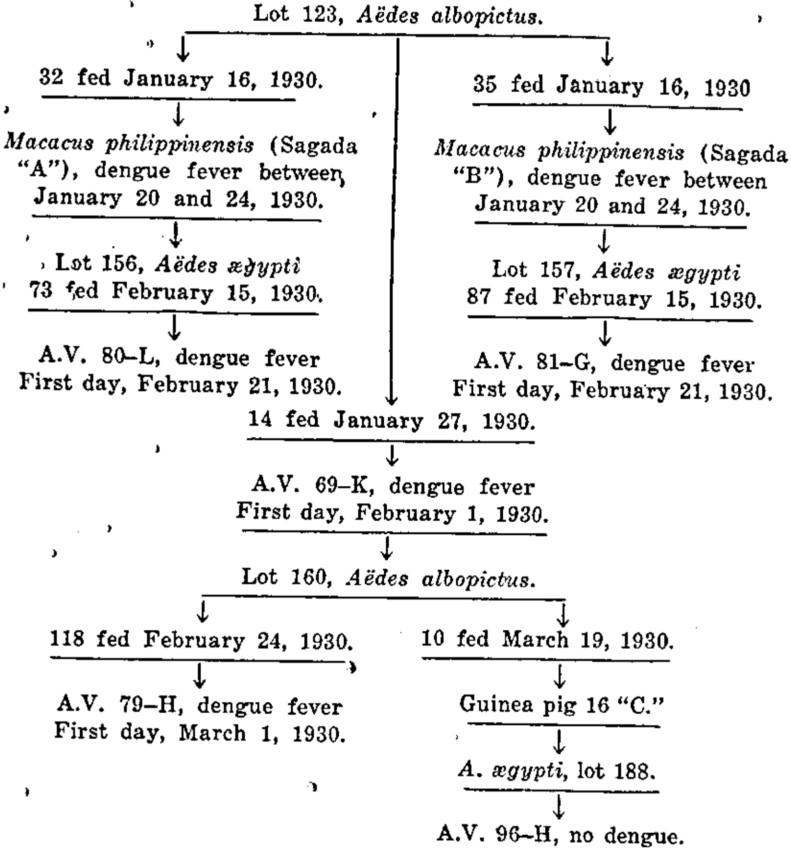
↓

A.V. 62–H, dengue fever
First day, December 2, 1929.

↓

Lot 128, *Aëdes albopictus.*

CHART 2.—*Continuation of the passage of "K" strain of dengue virus through Aëdes ægypti from July 11, 1929, to November 28, 1929, and after that date Aëdes albopictus of lots 128 and 160*—Continued.



Lot 123, *Aëdes albopictus.*

32 fed January 16, 1930.

Macacus philippinensis (Sagada "A"), dengue fever between January 20 and 24, 1930.

Lot 156, *Aëdes ægypti*
73 fed February 15, 1930.

A.V. 80–L, dengue fever
First day, February 21, 1930.

35 fed January 16, 1930

Macacus philippinensis (Sagada "B"), dengue fever between January 20 and 24, 1930.

Lot 157, *Aëdes ægypti*
87 fed February 15, 1930.

A.V. 81–G, dengue fever
First day, February 21, 1930.

14 fed January 27, 1930.

A.V. 69–K, dengue fever
First day, February 1, 1930.

Lot 160, *Aëdes albopictus.*

118 fed February 24, 1930.

A.V. 79–H, dengue fever
First day, March 1, 1930.

10 fed March 19, 1930.

Guinea pig 16 "C."

A. ægypti, lot 188.

A.V. 96–H, no dengue.

These potentially infected *A. albopictus* were designated lot 128 and were later used to transmit dengue to two Philippine monkeys (*Macacus philippinensis*) and to one human volunteer as described in the following experiments.

Experiment 9.—January 16, 1930. Thirty-two *A. albopictus* of lot 128 fed on a Philippine monkey *M. philippinensis* 30 (Sagada "A"). This monkey had been obtained from the mountains of northern Luzon and observed in a screened cage for a period of about two weeks prior to this test. While the monkey failed to show any recognizable symptoms of dengue, groups of normal *A. ægypti*, which fed on the animal January 20, 21, 22, 23, and 24 and were designated lot 156 later transmitted dengue (February 15, 1930) to a susceptible human volunteer (A.V. 80–L). Comment: The observations made in this experiment show that thirty-two *A. albopictus* of lot 128 produced dengue in a Philippine monkey.

Experiment 10.—January 16, 1930. Thirty-five *A. albopictus* of lot 128 fed on *M. philippinensis* 31 (Sagada "B"), which had been obtained from the mountains of northern Luzon and observed in a screened cage for a period of about two weeks prior to this test. While this monkey also failed to show any recognizable signs of dengue, it is apparent that infection occurred, since groups of normal *A. ægypti* fed on the animal January 20, 21, 22, 23, and 24, and designated lot 157, later transmitted dengue (February 15, 1930) to a susceptible human volunteer (A.V. 81–G). Comment: The observations made in this experiment show that the feeding of thirty-five *A. albopictus* of lot 128 produced dengue in a Philippine monkey.

Experiment 11.—January 27, 1930. Fourteen *A. albopictus* of lot 128 fed on a volunteer (A.V. 69–K) who had been used previously for a negative experiment January 13 and had been observed for fourteen days prior to this test. After an incubation period of five days, typical dengue fever developed at noon February 1, beginning with a chill followed by fever. The symptoms, which persisted for about five and one-half days, included headache, pain in various parts of the body, glandular enlargement, weakness, dizziness, fever, leukopenia, and a terminal rash. Comment: The feeding of fourteen infected *A. albopictus* of lot 128 on this volunteer was followed in five days by typical dengue fever.

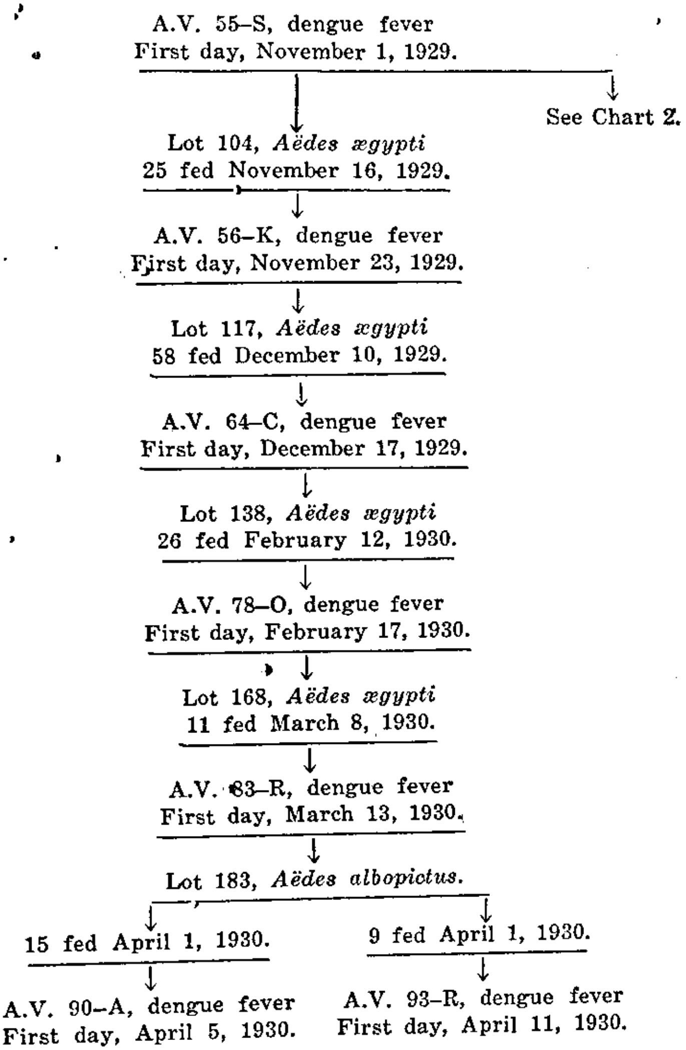
EXPERIMENTS WITH AËDES ALBOPICTUS, LOT 160

February 2, 1930, two hundred fifty normal *A. albopictus* took infective blood from an experimental case of dengue (A.V. 69–K) during the first day of the disease. These mosquitoes, designated lot 160, were later fed on one human volunteer and one guinea pig, as described in experiments 12 and 13.

Experiment 12.—February 24, 1930. One hundred eighteen *A. albopictus* of lot 160 fed on a volunteer (A.V. 79–H) who had been used previously for a negative experiment February 15, 1930, and had been observed for eight days prior to this test. After an incubation period of five days, typical dengue fever developed March 1, 1930, beginning with fever, nausea, and vomiting. The symptoms continued for about five days and were characterized by both primary and terminal rashes, fever, leukopenia, nausea, dizziness, headache, pains in eyes, back, chest, and joints, and some glandular enlargement. Comment: The feeding of one hundred eighteen *A. albopictus* of lot 160 on this volunteer was followed in five days by typical dengue fever.

Experiment 13.—March 19, 1930. Ten *A. albopictus* of lot 160 fed on a young guinea pig "C," which had been born in a mosquito-proof cage and kept there until the time of this test. Normal *A. ægypti* (lot 188) were fed on this guinea pig daily during the fifth to eighth days, inclusive, after it had been bitten by *A. albopictus* (lot 160). Six and twelve *A. ægypti* of lot 188 were tested on dengue-susceptible volunteer A.V. 96–H. Dengue did not develop. Comment: Either the guinea pig was immune or the mosquitoes, fed on the day when the virus was infective, had died before they were tested on the volunteer. This experiment was considered inconclusive.

CHART 3.—*Continuation of passage of "K" strain of dengue virus through Aëdes ægypti from November 1, 1929, to March 13, 1930, and after that date by Aëdes albopictus of lot 183.*



EXPERIMENTS WITH AËDES ALBOPICTUS, LOT 183

March 13, 1930, one hundred sixteen normal *A. albopictus* took infective blood from an experimental case of dengue (A.V. 83–R) during the first day of the disease. These mosquitoes, designated lot 183, were later used in transmission experiments with two human volunteers as described in experiments 14 and 15.

Experiment 14.—April 1, 1930. Fifteen *A. albopictus* of lot 183 fed on a volunteer (A.V. 90–A) who had been used previously for a negative experiment March 22, 1930, and had been observed for nine days prior to this test. After an incubation period of four and one-half days, dengue fever developed April 5, 1930.

Experiment 15.—April 1, 1930. Nine *A. albopictus* of lot 183 fed on a volunteer (A.V. 93–R) who had been used previously for a negative experiment March 22, 1930, and had been observed for nine days prior to this test. After an incubation period of ten days dengue fever developed April 11, 1930.

DISCUSSION

The results of these experiments prove that *Aëdes albopictus*, a common Oriental mosquito, is an effective dengue carrier, and make it necessary to reconsider the available evidence concerning the epidemiological importance of this species. From the observations made by the board and by others who have studied the mosquito, it is apparent that *A. albopictus* somewhat resembles *A. ægypti* in its choice of breeding places and in its habit of feeding on human beings mainly during the day. However, the former mosquito may require a few more days for development to the adult stage, and appears to be even more vicious and daring in biting man. The geographical distribution of *A. albopictus* given by Theobald,(5) Barraud,(6) and others indicates that this species is prevalent in many localities, including India, Formosa, the Philippine Islands, and other parts of the Orient where dengue is endemic. Cleland(8) and others mention the fact that *A. albopictus* has been suspected as a dengue vector on epidemiological grounds alone; while this mosquito was under grave suspicion during the 1915 dengue epidemic in Formosa.(7) Furthermore, observations made in Manila during 1929 indicate that this mosquito was especially prevalent during June and July, at the time of the highest incidence of dengue fever. From the evidence presented in this report, it is obvious that *A. albopictus* must be considered seriously as a factor in the dissemination of dengue fever.

SUMMARY AND CONCLUSIONS

1. Collections of larvæ and of adult mosquitoes from representative parts of the city indicate that *Aëdes albopictus* Skuse, like *A. ægypti*, is a common species in Manila, Philippine Islands.

2. A review of the literature indicates that *A. albopictus* is prevalent not only in the Philippines but throughout the Orient, and that this mosquito has been suspected as a possible vector of dengue.

TABLE 4.—*Transmission of dengue to man and monkeys by Aëdes albopictus.*

Experiment No.	Infective feeding.					
	Lot No.	Number fed.	Date fed.	Time fed.	Source of virus.	Day of dengue.
				a. m.		
1	29	80	July 16, 1929	10	A.V. 22-M	First.
2						
3						
4						
5						
6	48	54	Aug. 11, 1929	11.30	A.V. 27-P	Do.
7						
8	58	16	Sept. 6, 1929	9.30	A.V. 34-S	Do.
9	128	184	Dec. 4, 1929	11	A.V. 62-H	Second.
10						
11						
12	160	250	Feb. 2, 1930	9	A.V. 69-K	First.
14	183	116	Mar. 13, 1930	10.30	A.V. 83-R	Do.
15						

Experiment No.	Subsequent use.				
	Number fed.	Date fed.	Time fed.	On whom fed.	Onset of dengue.
			a. m.		
1	12	July 29, 1929	10	A.V. 23-B	a. m. Aug. 5, 1929.
2	8	July 29, 1929	10	A.V. 26-T	p. m. Aug. 4, 1929.
3	3	Aug. 6, 1929	11.30	A. V. 25-D	p. m. Aug. 12, 1929.
4	1	Aug. 6, 1929	11.30	A.V. 27-P	a. m. Aug. 11, 1929.
5	10	Aug. 7, 1929	11.15	A.V. 24-H	a. m. Aug. 12, 1929.
6	22	Aug. 26, 1929	10	A.V. 32-W	a. m. Aug. 31, 1929.
7	2	Aug. 31, 1929	10	A.V. 34-S	a. m. Sept. 5, 1929.
8	3	Oct. 2, 1929	11	A.V. 45-V	Sept. 7, 1929.
9	82	Jan. 6, 1930	9–11	*Macacus philippinensis* 30 (Sagada "A").	Between Jan. 20 and 24, 1929.[a]
10	85	Jan. 16, 1930	9–11	*Macacus philippinensis* 31 (Sagada "B").	Between Jan. 20 and 24, 1929.[a]
11	14	Jan. 27, 1930	10	A.V. 69-K	Feb. 2, 1930.
	8	Feb. 7, 1930	9	A.V. 72-E	(To test immunity.)
12	118	Feb. 24, 1930	10.30	A. V. 79-H	Mar. 1, 1930.
14	15	Apr. 1, 1930	9.30	A.V. 90-A	April 5, 1930.
15	9	Apr. 1, 1930	9.30	A.V. 93-R	April 11, 1930.

[a] Infection was proved in these monkeys by transferring the virus from them through *A. ægypti*, lots 156 and 157 to susceptible volunteers A.V. 80-L., A.V. 81-G, and A.V. 69-K.

3. During these experiments the transmission of dengue by this mosquito to twelve American soldiers and to two Philippine monkeys (*Macacus philippinensis*) proves that *A. albopictus* is an effective carrier of dengue.

4. In these successful transmission experiments with *A. albopictus* the intervals between the ingestion of infective blood and the tests on volunteers were from thirteen to fifty-four days.

5. Dengue resulted in the different experiments from the feeding of 1, 2, 3, 8, 8, 9, 10, 12, 14, 15, 22, 32, 35, and 118 infected *A. albopictus*, respectively.

6. *Aëdes albopictus* must be considered as an important agent in the dissemination of dengue.

7. In view of the fact that two distinct species of *Aëdes* are now known to be dengue vectors, it is reasonable to suspect that still others may be concerned in the spread of this disease.

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DENGUE TRANSMISSION EXPERIMENTS WITH CULEX QUINQUEFASCIATUS

Culex quinquefasciatus, formerly known as *Culex fatigans*, was the first mosquito to be incriminated as a dengue vector. Observations of early workers concerning the similarity between the distribution of dengue and the geographical distribution of this mosquito led to its use in transmission experiments. Graham,(1) who first established the fact that dengue can be transferred to man by mosquitoes, reported several successful experiments with *C. fatigans*. In his work, mosquitoes which had been captured inside the netting covering dengue patients were immediately placed inside the netting covering normal persons. Dengue occurred in three of four experimental volunteers after incubation periods of four, five, and six days. While the volunteers had agreed to remain in their homes throughout the experiments, Graham was apparently not sure on this point, as he made the following comment: "I still felt that these three men might have received the contagion in some other way." He then collected mosquitoes from inside the netting of a dengue patient, took them to a dengue-free village at an elevation of 3,000 feet, and gave them an opportunity to feed on two young men who lived in different parts of the town. After incubation periods of four and five days, respectively, both of these men developed dengue. No other case occurred in the village. Since there was only a short interval between the first and second stages of these experiments, it seems possible that in some instances an interrupted type of feeding might have occurred, resulting in a mechanical transfer of dengue-infected blood to the volunteers. However, uncertainty about the possibility of having included some "Stegomyia" with the lots of *Culex* used, has led to doubt concerning the identity of the mosquitoes responsible for the infections in his experiments.

Stimulated by Graham's observations and by the fact that this mosquito was commonly found in endemic dengue localities in the Philippines, Ashburn and Craig(2) (1907) used *Culex fatigans*, raised from the egg in their laboratory, in a series of transmission experiments. The procedure followed was to liberate the mosquitoes at night beneath the net covering a dengue patient isolated in a mosquito-proof tent. On the following morning the mosquitoes were collected and liberated inside a mosquito bar covering the experimental dengue volunteer who remained beneath the net until all the mosquitoes had disappeared. Four of nine volunteers exposed in this manner were satisfactorily bitten, and one of these four developed typical dengue. It was believed that this individual had not been exposed to dengue for some time prior to the experiment, and he was not allowed to leave the mosquito-proof covering until after the disease began. In this one successful experiment the mosquitoes which had fed on a patient September 11, 1906, were placed in the net with the volunteer on the following day, September 12, and dengue developed a little over four days later.

Cleland, Bradley, and McDonald(3) in 1916 performed four experiments using a slightly different technic in which they failed to transmit dengue with *Culex fatigans*. In the first series mosquitoes collected in the rooms of dengue patients April 3, 4, and 6, 1916, were given an opportunity to feed on two volunteers, "Mac" and "Joe," April 8, 9, and 11. Four mosquitoes took blood from "Mac" on the evening of April 8, while two, "several," and one bit J. O. S. on the respective days. Dengue did not develop. In the second series of experiments a volunteer "M" was bitten by twelve of these mosquitoes May 11, and by an unknown number May 12 and 13, and by at least twenty May 14. Then after adding fifty-three *Culex*, some of which had been caught in the houses of dengue patients, the lot was given access to a laboratory assistant, J. O. S., who was bitten by two mosquitoes May 15 and by —— May 18, 1916. Both of these men remained well. In discussing these four experiments the authors made the following comment: "We have not in our opinion shown conclusively that *Culex fatigans* may not also spread the disease though we think this unlikely." They also remarked concerning the experiments of Ashburn and Craig: "If their successful case arose from the bites of *Culex fatigans* and was not a case of natural infection, the most reasonable view to take is that in this instance the mosquitoes merely acted as infected lancets and not as true intermediate hosts." Siler, Hall, and Hitchens(4) in 1924 tried to infect seven susceptible volunteers with laboratory-bred *Culex quinquefasciatus*, which twelve to twenty-two days previously had fed on patients during the infective stage of dengue. The results were negative, while *A. ægypti,* which had been fed on infected blood from the same sources and later fed on the same volunteers used for the *Culex* experiments, produced dengue in each instance. Blanc and Caminopetros(5) in 1928 reported failure to transmit dengue with *Culex* mosquitoes, including *C. pipiens, C. apicalis,* and *C. laticinctus,* during experiments in which, after feedings of infective blood, the mosquitoes were kept five, ten, fifteen, eighteen, twenty-two, and thirty-four days before they were tested for infectivity.

In the present study the experiments were planned with a view to determining whether or not *C. quinquefasciatus* becomes infected after a feeding of dengue blood; and, if infected, whether or not it is able to transmit the disease to man after a period similar to that required by *A. ægypti.*

EXPERIMENTAL

Source of mosquitoes.—The two lots of *Culex quinquefasciatus* (Nos. 31 and 133) used in these experiments were selected from mosquitoes that emerged from larvæ collected in Manila and raised in the laboratory. Each mosquito conformed to the description of *C. quinquefasciatus* given by Dyar.(6)

Source of dengue virus.—The blood fed to the mosquitoes of these lots contained infective dengue virus. Lot 31: July 16, 1929, between 7 and 9 p. m., one hundred seven normal *C. quinquefasciatus* took full feedings of infective blood from an experimental case of dengue (A.V. 21–M), seven hours after the onset of the disease. The infectivity of the blood at this time was indicated by the fact that normal *A. ægypti,* which fed on the patient at 9.15 a. m., July 17 (lot 32), later transmitted dengue to a volunteer (A.V. 28–P). The *Culex* mosquitoes fed on this case and designated lot 31 were later used in experiments 1, 2, 3, 4, and 5. Lot 133: December 4, 1929, between 7 and 10.30 p. m., fifty-two normal *C. quinquefasciatus* took full feedings of blood from an experimental case of dengue (A.V. 60–S) during the first day of the disease. These mosquitoes were used in experiment 6.

Experiment 1.—Attempt to determine whether or not infective virus remains in Culex quinquefasciatus four days after the ingestion of dengue blood. July 20, 1929, 1.15 p. m. Twenty-five *C. quinquefasciatus* of lot 31, which had *fed on infective blood four days previously* were killed, macerated with sterile sand in a porcelain mortar, suspended in physiologic salt solution, and filtered through a preliminary type of Mandler filter. Three cubic centimeters of the filtrate, representing about eighteen of the mosquitoes, was injected subcutaneously into a susceptible volunteer (A.V. 28–P). Dengue failed to develop from this inoculation. Comment: The evidence was not considered sufficient to prove that these mosquitoes were not infected, because similar negative results were also obtained at times even with filtrates obtained from suspensions of *A. ægypti* known to be infective. Therefore, no definite conclusion could be drawn from this experiment.

The next four experiments were performed in order to determine the ability of *C. quinquefasciatus* to transmit dengue by biting thirteen, thirty-three, and forty-five days after they had ingested infective blood.

Experiment 2.—July 29, 1929, 7 to 9 p. m. Seven *C. quinquefasciatus* of lot 31, *which had taken infective blood thirteen days previously*, fed on a susceptible volunteer (A.V. 24–H). Dengue did not develop. August 7 this volunteer was tested for susceptibility with an infective lot of *A. albopictus* (No. 29), and developed dengue August 12. Comment: In this experiment *C. quinquefasciatus*, which had ingested infective blood thirteen days previously, failed to transmit dengue by biting.

Experiment 3.—July 29, 1929, 7 to 9 p. m. Four *C. quinquefasciatus* of lot 31, *which had taken infective blood thirteen days previously*, fed on a susceptible volunteer (A.V. 25–D). Dengue did not develop in thirteen days. This volunteer was tested for susceptibility with an infective lot of *A. albopictus* (No. 29) August 6, and developed dengue August 12, 1929. Comment: This experiment indicates that *C. quinquefasciatus*, which had ingested infective blood thirteen days previously, failed to transmit dengue.

Experiment 4.—August 18, 1929, 6 to 9 p. m. Ten *C. quinquefasciatus* of lot 31, *which had ingested infective blood thirty-three days previously*, fed on a susceptible volunteer (A.V. 31–D). Dengue did not develop in eleven days. This volunteer was tested for susceptibility with an infective lot of *A. ægypti* (No. 35) August 26, and developed dengue four days later, August 30, 1929. Comment: As the interval between the feeding of *Culex* on this volunteer and the beginning of dengue was longer than the usual incubation periods observed in dengue, there was no reason to suppose that the infection was due to *Culex*, particularly since infective *A. ægypti*, lot 35, had fed in the meantime. However, in the next experiment it was planned to observe the volunteer for a longer period before applying the test for susceptibility.

Experiment 5.—August 30, 1929, 6 to 9 p. m. Five *C. quinquefasciatus, which had taken infective blood forty-five days previously*, fed on a susceptible volunteer (A.V. 33–B). Dengue did not develop in eighteen days. This volunteer was purposely held for a longer period than usual before applying the test for susceptibility, in order to eliminate the possibility of infection after a prolonged incubation period. The test for susceptibility was made September 14 with an infective lot of *A. ægypti* (No. 49), and dengue followed after an incubation period of five days, September 18, 1929. Comment: In this experiment *C. quinquefasciatus*, which had ingested infective blood forty-five days previously, failed to transmit dengue by biting.

Experiment 6.—To determine whether infective virus remains in Culex quinquefasciatus fourteen days after the ingestion of dengue blood. December 18, 1929. Thirty-eight *C. quinquefasciatus* of lot 133 were macerated, mixed with 2.5 cubic centimeters of a normal guinea pig's blood, placed in a shallow cell under a piece of chicken skin, and fed to one hundred forty normal *A. ægypti*. The latter mosquitoes, designated *A. ægypti* lot 104, were tested after thirty-five days by feeding on a susceptible volunteer (A.V. 75–P), who did not develop dengue during an observation period of sixteen days. February 8 dengue fever developed from the feeding of dengue-infected *A. ægypti* of lot 138, February 3, 1930. Comment: As normal *A. ægypti* may be infected with dengue virus by feeding on similarly prepared suspensions of infected *Aëdes*, it is believed that the negative results obtained in this experiment indicate that *C. quinquefasciatus* of lot 133 were not properly infected fourteen days after they had ingested blood from a dengue patient.

DISCUSSION

Groups of *Culex quinquefasciatus* which had fed on infective blood thirteen to forty-five days previously failed to transmit dengue when tested on susceptible volunteers. Similar negative observations have been reported by Siler, Hall, and Hitchens(4) and by others who have investigated the possibility of dengue transmission by this mosquito under conditions similar to those required for *A. ægypti*.

Attempts were also made to determine the length of time during which dengue virus remains alive after ingestion by *Culex*, and to determine whether this mosquito may become permanently infected as does *A. ægypti*, but is unable to transmit the virus to man during feeding because of some physiological or anatomical peculiarity. At first it was considered probable that these questions might be answered by injecting susceptible volunteers with bacteria-free filtrates of suspensions of *Culex* killed at different intervals after they had fed on blood containing infective dengue virus. However, because of the irregular results obtained in filtration experiments with suspensions of *A. ægypti* known to have been infective, the method was abandoned as unreliable. The later development of a technic by which normal *A. ægypti* were infected with dengue virus by feeding on suspensions of infective *A. ægypti* in normal blood, led to the use of a similar technic for testing *C. quinquefasciatus* for infectivity. Such experiments were begun with two groups of *Culex*, which had fed on dengue blood twenty-four hours and fourteen days previously. Unfortunately the former experiment was not completed, due to loss of the *A. ægypti* that fed on the suspension of *Culex* which had ingested blood containing dengue virus twenty-four hours previously. However, the latter experiment (No. 6) was carried through satisfactorily. The result was negative, indicating that either dengue virus was not present in the *Culex* mosquitoes used in the suspension or, if present, the amount was so small or the virus so attenuated that the infection could not be transferred through *A. ægypti* to man. Whatever the real explanation, it is apparent that *C. quinquefasciatus* may feed on a case of dengue during the infective stage of the disease and again feed on susceptible individuals thirteen to forty-five days later without transmitting dengue.

These observations naturally have no application to the possibility of transferring dengue virus mechanically by *Culex* or other blood-sucking insects that are interrupted while feeding on infective patients and immediately afterwards complete their meal of blood on susceptible individuals. The subject of mechanical transmission is discussed in a separate chapter.

SUMMARY AND CONCLUSIONS

1. In four experiments *C. quinquefasciatus*, which had fed on infective blood thirteen to forty-five days previously, failed to transmit dengue by biting susceptible volunteers.

2. A volunteer injected subcutaneously with the "Mandler" filtrate of a saline suspension of *C. quinquefasciatus*, which four days previously had fed on infective dengue blood, failed to develop dengue.

3. Normal *A. ægypti*, fed on a suspension of *C. quinquefasciatus* fourteen days after the latter had ingested dengue blood, failed to transmit dengue to a susceptible volunteer. Therefore, it seems probable that within fourteen days or less after *C. quinquefasciatus* has ingested infective blood the virus in this mosquito either dies or loses its ability to infect either man or *Aëdes* mosquitoes.

4. If *C. quinquefasciatus* is able to transmit dengue to man it is believed that this probably occurs as a result of mechanical transfer of infected blood during interrupted feeding.

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MECHANICAL TRANSMISSION OF DENGUE VIRUS THROUGH "INTERRUPTED FEEDING" BY CULEX QUINQUEFASCIATUS AND AËDES ÆGYPTI

It is not unusual for mosquitoes interrupted while feeding on one individual to attempt to complete their meal of blood on the same person or on others. That mosquitoes are rarely allowed to take a complete feeding of blood from a single person cannot be questioned, as the discomfort associated with the insertion of the proboscis usually leads to attempts to destroy the insect, an act which is often unsuccessful and usually results only in interrupting the feeding. While the mosquito may attempt to feed again on the same individual, it may attack some other person. Thus, if an organism of sufficient virulence should be present in the blood of the first individual bitten, it is not unreasonable to suspect that in some instances it might be carried mechanically on or in the proboscis of the mosquito to other individuals and that in this way it might be possible to transmit certain blood infections mechanically. The possibility of such mechanical transmission of dengue by mosquitoes has been suggested and discussed by various investigators; while Graham(1) and Ashburn and Craig(2) have reported positive experiments with *Culex quinquefasciatus*, in which dengue transmission apparently resulted from interrupted feeding of the mosquitoes on dengue patients followed immediately by refeeding on susceptible persons.

EXPERIMENTAL

The possibility of transmitting dengue in this manner was investigated by allowing *Culex quinquefasciatus* and *Aëdes ægypti* to partially fill with infective blood from experimentally induced cases of dengue, after which the mosquitoes were immediately transferred to susceptible human volunteers and allowed to complete the interrupted feedings.

MECHANICAL TRANSMISSION OF DENGUE VIRUS BY CULEX QUINQUEFASCIATUS

Experiment 1.—A susceptible volunteer (A.V. 54–K) admitted October 21, 1929, was observed in a screened cubicle for ten days before the exper-

MECHANICAL TRANSMISSION OF DENGUE VIRUS THROUGH "INTERRUPTED FEEDING" BY CULEX QUINQUEFASCIATUS AND AËDES ÆGYPTI

It is not unusual for mosquitoes interrupted while feeding on one individual to attempt to complete their meal of blood on the same person or on others. That mosquitoes are rarely allowed to take a complete feeding of blood from a single person cannot be questioned, as the discomfort associated with the insertion of the proboscis usually leads to attempts to destroy the insect, an act which is often unsuccessful and usually results only in interrupting the feeding. While the mosquito may attempt to feed again on the same individual, it may attack some other person. Thus, if an organism of sufficient virulence should be present in the blood of the first individual bitten, it is not unreasonable to suspect that in some instances it might be carried mechanically on or in the proboscis of the mosquito to other individuals and that in this way it might be possible to transmit certain blood infections mechanically. The possibility of such mechanical transmission of dengue by mosquitoes has been suggested and discussed by various investigators; while Graham(1) and Ashburn and Craig(2) have reported positive experiments with *Culex quinquefasciatus*, in which dengue transmission apparently resulted from interrupted feeding of the mosquitoes on dengue patients followed immediately by refeeding on susceptible persons.

EXPERIMENTAL

The possibility of transmitting dengue in this manner was investigated by allowing *Culex quinquefasciatus* and *Aëdes ægypti* to partially fill with infective blood from experimentally induced cases of dengue, after which the mosquitoes were immediately transferred to susceptible human volunteers and allowed to complete the interrupted feedings.

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iment. October 31, 1929, between 9 and 10.30 p. m., one hundred normal *Culex quinquefasciatus*, designated lot 97, were permitted to take partial feedings of infective blood from an experimental case of dengue (A.V. 55–S) during the first day of the disease. These mosquitoes were immediately transferred to the susceptible volunteer (A.V. 54–K) on whom fifty-four of them resumed feeding and became completely filled with blood. After an incubation period of eleven and one-half to thirteen days this volunteer developed dengue fever. The temperature reached 99.4° F. at noon November 12, while the patient first complained of illness on the evening of November 13. The symptoms as noted in the protocol of this volunteer were typical of dengue. Comment: In this experiment dengue apparently was transmitted to man mechanically through interrupted feeding by *C. quinquefasciatus*.

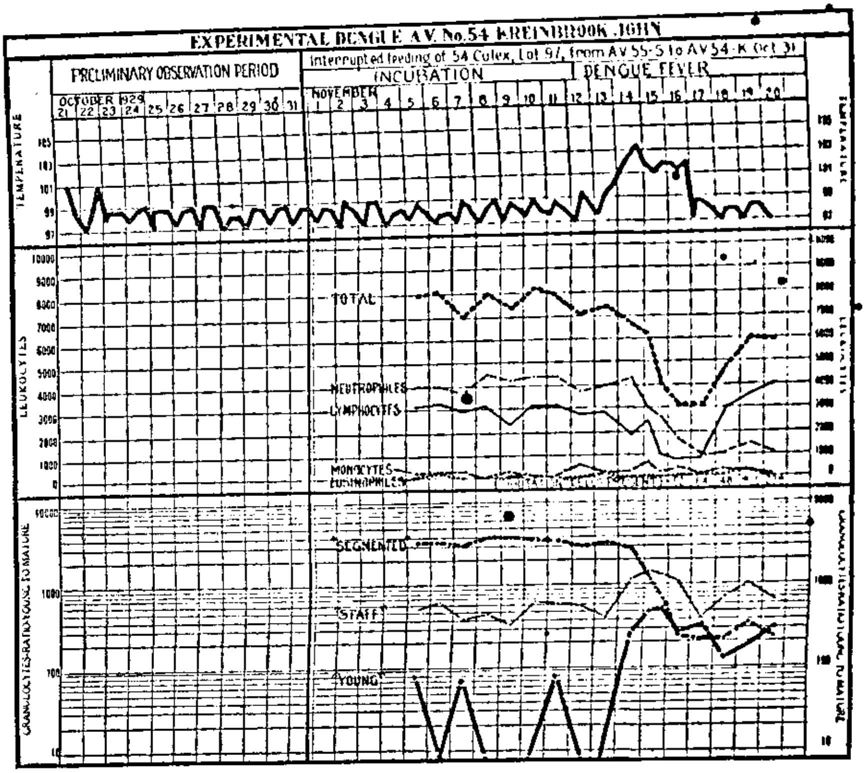

FIG. 10. Experimental dengue in American volunteer 54.

As the positive result obtained in the first experiment indicated that dengue virus might be transferred mechanically when large numbers of mosquitoes took part in the feeding, the following experiments were performed to determine whether similar results might be obtained when small numbers of mosquitoes were employed.

Experiment 2.—December 4, 1929, 6.30 to 7.30 p. m. Eight *C. quinquefasciatus* called lot 132, which had partially filled with infective blood from A.V. 60–S during the first day of dengue, immediately completed feeding on a susceptible volunteer (A.V. 58–S). This man did not develop dengue during fifteen days of subsequent observation in the ward. Because of the Christmas holidays, the volunteer was allowed to leave the ward before a test was made to determine his susceptibility to dengue, and it was not possible to do this until three months later. No history could be obtained of any illness during this period. March 31, 1930, twenty-six infected *A. ægypti* fed on him, after which he remained well for a period of at least two weeks and, therefore, was considered to be immune to dengue. Comment: The results of this experiment were inconclusive, due to the fact that the volunteer used to test the infectivity of the mosquitoes was immune.

Experiment 3.—February 21, 1930. Twenty-one of twenty-six *C. quinquefasciatus* called lot 170, which had partially filled with infective blood from A.V. 80–L between 6.30 and 9.30 p. m. on the first day of dengue, completed feeding before 10.30 p. m. on a susceptible volunteer (A.V. 82–F). Dengue did not develop during an observation period of eighteen days; however, after one hundred eight infective *A. ægypti* of lot 167 had fed March 4, dengue followed March 11, 1930. Comment: In this experiment twenty-one *C. quinquefasciatus,* which had partially fed on dengue blood and immediately refed on a susceptible volunteer, failed to transmit dengue.

Experiment 4.—March 13, 1930. Twelve of twenty-six normal *C. quinquefasciatus,* designated lot 185, which had taken partial feedings of infective blood from a patient (A.V. 83–R) between 7 and 10.30 p. m. on the first day of dengue, completed feeding within an hour on a susceptible volunteer (A.V. 85–T). Dengue did not develop during eighteen days observations. Infected *A. ægypti* of lot 164 were fed March 26, and after six and one-half days dengue occurred April 1, proving that the volunteer was not immune. Comment: In this experiment the refeeding of twelve *C. quinquefasciatus,* immediately after they had ingested dengue blood, failed to cause the disease in a susceptible volunteer.

Experiment 5.—March 13, 1930. Eight of twenty-six *C. quinquefasciatus* called lot 185, which had taken infective blood from a patient (A. V. 83–R) between 7 and 10.30 p. m. on the first day of dengue, completed feeding between 9 and 11 p. m. on a susceptible volunteer (A.V. 86–B). This man remained well for seventeen days. However, after being bitten by twenty-three *A. ægypti,* lot 165, March 26, dengue developed March 31. Comment: In this experiment the refeeding of eight *C. quinquefasciatus* within a few minutes after they had ingested infective dengue blood failed to cause the disease in a susceptible volunteer.

MECHANICAL TRANSMISSION OF DENGUE VIRUS BY AËDES ÆGYPTI

Experiment 6.—March 24, 1930. Two hundred normal *A. ægypti,* called lot 189, which at 10.30 a. m. had taken partial feedings of infective blood from a volunteer (A.V. 87–R) during the first day of dengue, were transferred immediately to a susceptible volunteer (A. V. 94–K). More than one hundred twenty-five of the mosquitoes refed and became filled with blood. After an incubation period of seven to nine days this volunteer developed dengue fever. Comment: In this experiment dengue was apparently transmitted to man through the interrupted feedings of *A. ægypti.*

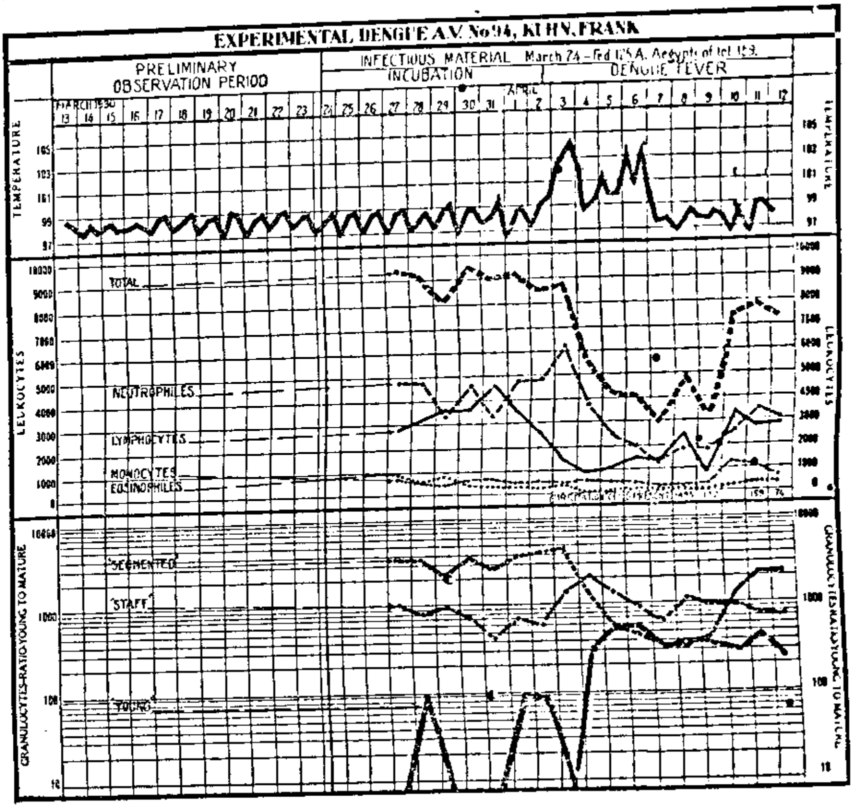

FIG. 11. Experimental dengue in American volunteer 94.

DISCUSSION

The results obtained in these experiments indicate that during the infective period of dengue fever, virus may be carried from patients to susceptible individuals by immediate mechanical transfer on or in the proboscis of *C. quinquefasciatus* or *A. ægypti.* However, such transfer of virus occurred only in experiments 1 and 6, in which relatively large numbers of mosquitoes, fifty-four and one hundred twenty-five, respectively, completed their feedings on susceptible volunteers; while in similar experiments performed with eight, twelve, and twenty-

TABLE 5.—*Mechanical transmission of dengue virus by interrupted feedings of Culex quinquefasciatus and Aëdes ægypti.*

Experiment No.	Date.	Mosquitoes allowed partial feedings of infective blood.					
		Experimental case of dengue.		Mosquitoes that took blood.			
		Volunteer.	Day of the disease.	Species.	Lot No.	Number fed.	Time fed.
1	Oct. 31, 1929	A.V. 55-S	First	*Culex quinquefasciatus.*	97	100	9-10.30 p. m.
2	Dec. 4, 1929	A.V. 60-S	do	do	132	8	6.30-7.30 p. m.
3	Feb. 21, 1930	A.V. 80-L	do	do	170	26	6.30-9.30 p. m.
4	Mar. 13, 1930	A.V. 83-R	do	do	185	26	7-10.30 p. m.
5	Mar. 13, 1930	A.V. 83-R	do	do	185	26	7-10.30 p. m.
6	Mar. 24, 1930	A.V. 87-R	do	*Aëdes ægypti*	189	160	10.30 a. m.

Experiment No.	Date.	Same mosquitoes refed on susceptible volunteers.			
		Time fed.	Number fed.	Volunteer.	Results.
1	Oct. 31, 1929	10.30-11.30 p. m.	54	A.V. 54-K	Dengue fever (in 11½ to 13 days).
2	Dec. 4, 1929	6.45-8 p. m.	8	A.V. 58-S	Immune.
3	Feb. 21, 1930	6.45-10.30 p. m.	21	A.V. 82-F	Negative.
4	Mar. 13, 1930	8-11 p. m.	12	A.V. 85-T	Do.
5	Mar. 13, 1930	8-11.00 p. m.	8	A.V. 86-B	Do.
6	Mar. 24, 1930	10.45 a. m.	125	A.V. 94-K	Dengue fever (in 7 to 9 days).

one mosquitoes the results were negative. These results indicate either that many individual mosquitoes failed to mechanically carry infective blood or virus; or that each mosquito used carried to the volunteer such minute amounts of the patient's blood that large numbers of mosquitoes feeding simultaneously were necessary in order to transfer sufficient virus to cause dengue infection in man. From the rather long incubation period it may be suspected that even in the successful experiments only small amounts of dengue virus were injected into the volunteers. For example, after the refeeding of fifty-four *C. quinquefasciatus* dengue was not apparent until eleven and one-half to thirteen days had elapsed; while after the refeeding of over one hundred twenty-five *A. ægypti* dengue occurred after seven to nine days. In this connection it is of interest to note that three volunteers, inoculated with Mandler or Berkefeld filtrates of suspensions of infected mosquitoes, also had rather long incubation periods, which were believed to have resulted from the small amounts of virus injected. Whatever the real explanation may be, the experimental results indicate that relatively large numbers of mosquitoes are required for mechanical transmission of dengue or that it is rarely transmitted in this manner by individual mosquitoes. As it seems probable that such favorable conditions rarely occur naturally, it may be assumed that mechanical transmission of dengue by "interrupted feeding" of *C. quinquefasciatus* or *A. ægypti* can occur, but that it is probably of slight importance in the natural dissemination of dengue.

SUMMARY AND CONCLUSIONS

1. The virus of dengue was transferred mechanically by (*a*) fifty-four *Culex quinquefasciatus* and by (*b*) one hundred twenty-five *Aëdes ægypti*, which were interrupted while feeding on infective dengue blood and immediately allowed to complete feeding on susceptible volunteers.
2. The periods of incubation before dengue was recognized in these two volunteers were eleven and a half to thirteen days in one case and seven to nine days in the other.
3. Three similar transmission experiments in which only eight, twelve, and twenty-one *C. quinquefasciatus* were used resulted negatively.
4. These experiments indicate that relatively large numbers of mosquitoes are necessary for the transmission of dengue in this manner, and for this reason it is considered probable that such mechanical transmission rarely occurs in nature.

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OBSERVATIONS TO DETERMINE WHETHER OR NOT DENGUE VIRUS CAN PENETRATE NORMAL HUMAN SKIN

While the usual method of transmission of dengue by certain insect vectors is now well recognized, it was considered of some practical interest to determine whether or not the virus of dengue, like certain other viruses and bacteria, is able to penetrate normal human skin and in this way cause infection.

Such direct passage of the virus if it occurred would, of course, make it possible to contract dengue by contamination of the skin with blood or other virus-containing materials from dengue patients, with the crushed bodies of dengue-infected insects, or with the infective excretions of insects. The chronic and prolonged carrier state developed in *Aëdes ægypti* after infection with dengue virus allows one at least to speculate concerning the possibility that other species of blood-sucking insects, after feeding on dengue blood, might also become infected, even though anatomical or biological differences interfered with their transmission of the infection to man again during feeding. If such chronic dengue infections should occur in other insects, the possibility of transferring the virus to man by crushing them on the skin or by contamination of the skin with their excretions becomes a problem of practical importance.

In this connection the negative experiments of Ashburn and Craig,(1) who in 1907 attempted to infect eight healthy men by contact with dengue patients and constant exposure to fomites, are of considerable interest. They concluded that dengue is not a contagious disease. Cleland, Bradley, and McDonald(2) (1919) failed to cause dengue by application of infective serum to the scarified skin of a volunteer or by the application of infective materials to the nasal mucous membranes. However, they obtained a doubtful result when infective material was used as a throat gargle and then swallowed.

While these observations indicate that the virus of dengue as it exists in infective blood is probably not able to penetrate even the abraded human skin, recent reports concerning the penetration of the skin of monkeys by yellow-fever virus from infective mosquitoes suggested the advisability of further work with dengue along similar lines.

In 1928, it was observed by Bauer and Hudson(3) that the virus of yellow fever, which in many ways behaves similarly to dengue virus, when present in sufficient amount in the blood of animals can penetrate the intact skin of rhesus monkeys and produce infection. These workers were also able to infect a monkey with yellow fever by applying to the scarified skin one drop of a suspension prepared by macerating ten infected *Aëdes ægypti* in a mortar with 1 cubic centimeter of salt solution. However, similar amounts of this same suspension rubbed on the shaven skin of a second monkey and on the unbroken skin of another failed to produce the disease in both tests.

Marchoux(4) (1929) observed that members of the French Yellow Fever Commission in Brazil, during 1903, performed about one hundred autopsies with their bare hands, yet none of them became infected. However, as the virus practically always disappears from the blood and organs before death, this does not prove that yellow-fever virus cannot penetrate the skin. He also reported experiments in which applications of infective defibrinated blood to the conjunctiva and skin of animals failed to produce the disease; while emulsions of infected liver instilled into the conjunctival sac or placed on the excoriated skin did cause yellow fever.

Aragao and Lima(5) (1929) reported that yellow-fever virus in the fæces of infected *Aëdes ægypti,* when applied either to the intact conjunctiva or the unbroken skin of susceptible monkeys, caused the disease.

EXPERIMENTAL

In order to determine whether or not penetration of the skin by dengue virus is possible, and if so whether this might be of practical importance in the natural transmission of the disease, the following experiments were performed in which materials from mosquitoes infected with dengue were rubbed on the skin of susceptible human volunteers.

Experiment 1.—July 11, 1929. Two *Aë. ægypti* of lot 25, which lot had been proved to be infected with dengue, were stunned by jarring, and were immediately crushed on the inner portion of the middle third of the right arm of a susceptible human volunteer (A.V. 26–T). By means of the rounded end of a glass test tube the crushed mosquito bodies were then rubbed thoroughly over the intact skin. The volunteer, who had already been observed in a screened cubicle during a preliminary observation period of eleven days, did not develop dengue from this experiment. The volunteer's susceptibility to dengue was demonstrated twenty-three days later, when the feeding on him of eight dengue-infected *A. albopictus* of lot 29, July 29, resulted in dengue August 4. Comment: The crushed bodies of two presumably dengue-infected *A. ægypti* rubbed into the intact skin of a susceptible human volunteer failed to produce dengue in this volunteer.

Experiment 2.—July 11, 1929. Two mosquitoes from a dengue-infected lot of *A. ægypti* (lot 25) were killed and crushed in a similar manner on the skin of a susceptible human volunteer (A.V. 27–P). After the mosquitoes had been well rubbed in, the skin was abraded by the volunteer, who had been instructed to scratch the area as though it itched. As a result, serum and a trace of blood exuded from the scratches. The volunteer, who had had the usual preliminary observation period before the test, did not develop dengue from this procedure. His susceptibility was proved one month later when he developed dengue August 11, five days after being bitten by one infected *A. albopictus* of lot 29. Comment: Dengue virus from the crushed bodies of two presumably dengue-infected *A. ægypti*, scratched and rubbed into the abraded skin of a susceptible human volunteer, failed to cause dengue.

Experiment 3.—October 26, 1929. Eleven mosquitoes from dengue-infected *A. ægypti* lot 71 were allowed to feed on a volunteer (A.V. 55–S). After an incubation period of five days, dengue fever developed, proving that this portion of lot 71 contained infected mosquitoes. October 28, 1929, or two days after feeding on volunteer 55–S, all of these eleven mosquitoes were killed, ground with pyrex glass "sand" in a sterile porcelain mortar, and suspended in 1 cubic centimeter of physiologic salt solution. This saline suspension of mosquitoes and dengue virus was immediately applied to the scarified skin of a human volunteer (A.V. 56–K) in the following manner. With a sterile needle three linear scratches, each about 2 centimeters in length, were made in the thin skin over the upper anterior aspect of the forearm. While no blood was drawn, small amounts of serum appeared in the abrasions. Then about 0.2 cubic centimeter of the saline suspension of dengue virus was rubbed into each of two of the abrasions, leaving one uninoculated for control observations on any local reaction that might have developed. There was no local reaction nor did this procedure cause dengue. The volunteer was proved susceptible to the disease twenty-six days later when, November 23, 1929, dengue resulted from twenty-five *A. ægypti* of lot 104 which had fed on him seven days earlier. Comment: Four-tenths (0.4) of a cubic centimeter of a saline suspension representing approximately four dengue-infected *A. ægypti* rubbed into the abraded skin of a human volunteer failed to cause dengue.

DISCUSSION

Ashburn and Craig(1) in 1907 exposed healthy men to the fomites of dengue patients in a manner similar to the experiments of Walter Reed and his coworkers with yellow fever. Since eight volunteers exposed in this way failed to develop dengue, it was concluded that dengue could not be transferred by contact with patients. Cleland, Bradley, and McDonald(2) (1919) failed to transmit dengue when infective material was applied to the nasal mucous membranes or when dengue-infective serum was applied to the scarified skin.

The negative results obtained in our experiments do not, of course, prove that dengue virus is unable to penetrate the skin under all conditions. For example, the concentration of virus in the materials used may be an important factor, as was suggested by the work of Bauer and Hudson(3) with yellow-fever virus. These investigators observed that when blood drawn from animals at the beginning of yellow fever was applied to the unbroken skin of monkeys, infection resulted; while applications of blood that had been drawn later in the disease and, therefore, presumably contained less virus failed. Furthermore, when saline suspensions of infected mosquitoes were used yellow fever occurred only when the material was applied to abraded skin.

However, in the present work with dengue, it may be presumed that the materials used contained sufficient virus to cause infection if penetration of the skin had occurred, since two mosquitoes from a known infected lot were used in both experiments 1 and 2, while the saline suspension used in experiment 3 represented the equivalent of at least four and four-tenths mosquitoes known to have been infected.

CONCLUSIONS

1. In these experiments dengue virus from infected *Aëdes ægypti,* rubbed on both the intact and the excoriated skin of susceptible human volunteers, failed to penetrate the skin and cause dengue. Therefore, it seems that contamination of the skin by dengue virus from crushed *A. ægypti* or from other insects in which the virus might exist, is probably not a factor in the natural spread of dengue.

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OBSERVATIONS ON THE POSSIBILITY OF HEREDITARY TRANSMISSION OF DENGUE FROM INFECTED FEMALE AËDES ÆGYPTI THROUGH THE EGG TO THE OFFSPRING

The possibility of the transmission of dengue virus through the eggs of infected female *Aëdes ægypti* to their offspring, as the organism of Rocky Mountain spotted fever may be passed through the eggs of its vector, *Dermacentor andersoni*, was reconsidered, since such hereditary transfer, if proved, should be of great importance to those interested in the control of dengue.

In 1905, Marchoux and Simond,(1) in an attempt to find an explanation for the reappearance of yellow fever in places from which the disease had apparently disappeared, conducted experiments to determine whether or not this might be caused by mosquitoes that had inherited the infection by passage of the virus of yellow fever through the egg. According to their report they succeeded, in one instance, in producing yellow fever with mosquitoes that had hatched from the eggs of infected mosquitoes. However, Phillips(2) injected two hundred eggs laid by yellow-fever-infected mosquitoes into six normal *Macacus rhesus* monkeys, with negative results. In five biting and two injection experiments with progeny of the same infective lot of mosquitoes, using seven normal monkeys, the results were also negative. Consequently, it was concluded that under the conditions of his experiments hereditary transmission of yellow fever by *A. ægypti* was improbable.

Siler, Hall, and Hitchens(3) investigated this possibility with mosquitoes infected with dengue. They collected the eggs laid by *A. ægypti* that had been proved to be infected with dengue virus; and fed the adults raised from these eggs on sugar solutions for periods of three to forty-two days, after which they were allowed to feed on three human volunteers. Eight days later the various lots were again applied to the same volunteers with negative results. After another period of eight days the volunteers were tested to determine their susceptibility by exposure to lots of infective *A. ægypti*. It was shown that one of the volunteers was immune to dengue, but two were susceptible. The following statement was made concerning the results: "We believe the conclusion is justified that the virus of dengue fever does not pass from the infected *Aëdes* through its eggs to the next succeeding generation."

EXPERIMENTAL

It was considered possible that even if dengue virus should pass from the infected female through the egg to the offspring, the latter might not be able to transmit the infection to man until twelve or more days had elapsed following the first feeding of normal blood. With this possibility in mind, the following experiment was performed.

Experiment 1.—December 15, 1928, fifty normal *Aëdes ægypti* took full feedings of infective blood from a case of dengue (Keller) during the second day of the disease and were designated lot 1. This was the first passage of the strain of dengue virus used throughout the various investigations made by the present board. Lot 1 *A. ægypti* transmitted dengue by feeding December 31, 1928, on two susceptible volunteers (A.V. 1–H and A.V. 2–M). Between January 4 and 8, 1929, or from twenty to twenty-four days after the infective feeding on Keller, the mosquitoes of lot 1 laid eggs which were collected and placed in a jar of water. Between January 15 and 20, 1929, the adult mosquitoes emerged and were fed on the blood of a normal guinea pig as follows: January 21, twelve fed; January 22, nineteen fed; January 23, eighteen fed, and January 24, eleven fed; making a total of sixty mosquitoes, which were combined and designated *A. ægypti* lot 8.

February 12, or from nineteen to twenty-two days after feeding on normal guinea-pig blood, forty-eight mosquitoes of lot 8 took full feedings of blood from a susceptible volunteer (A.V. 4–Y). Dengue did not develop during an observation period of thirteen days. However, after being tested for susceptibility with a known infective lot of *A. ægypti* (No. 2) February 19, 1929, this volunteer had dengue fever seven and one-half days later, on the evening of February 26, 1929.

CONCLUSION

The negative result obtained in this experiment agrees with the observations reported by Siler, Hall, and Hitchens and indicates that dengue virus is not transmitted from infected female *A. ægypti* through the egg to the offspring.

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EXPERIMENTAL ATTEMPTS TO TRANSMIT DENGUE VIRUS DIRECTLY TO LARVÆ OF NORMAL AËDES ÆGYPTI

The extreme susceptibility of adult *Aëdes ægypti* to infection with dengue virus, and the long chronic carrier state that follows infection of this mosquito, suggested the possibility that *A. ægypti* might also be infected during the larval stage by ingestion of dengue virus, added intentionally or accidentally to the fluids in which they are developing, and that if such infection were acquired it might persist throughout the adult stage of the mosquito. If it were possible to infect *A. ægypti* larvæ in such a manner, the question would, of course, arise as to the possibility of transferring dengue virus in nature directly from infected adult mosquitoes to normal larvæ by the accidental contamination of small breeding places, with the infective secretions or the dead bodies of the former. Such an occurrence would indicate the possibility of maintaining the virus of dengue in endemic localities by mosquitoes alone, without the necessity of alternate passages through vertebrate hosts.

No reference was found in the available literature to such investigations with dengue. However, according to Wittingham,(1) in 1924 the Royal Air Force Sandfly Commission observed that larvæ of *Phlebotomus* which were obtained from adults infected with the virus of sand-fly fever and bred under sterile conditions, except that they may have ingested the dejecta or dead remains of the parent flies, became infected after a preliminary feeding of normal human blood, and the adults that developed from these larvæ were able to transmit the disease to man.

EXPERIMENTAL

In these studies, eggs from normal *Aëdes ægypti* were placed in fruit jars containing 100 to 500 cubic centimeters of tap water. After the eggs had hatched, thin slices of ripe banana were added to each jar as food for the larvæ. At various intervals thereafter, suspensions of adult *A. ægypti* infected with dengue virus were added. The adult mosquitoes that developed from these larvæ were later tested for infectivity by feeding on susceptible human volunteers.

Experiment 1.—August 9, 1929. About one hundred recently hatched *A. ægypti* larvæ were placed in a fruit jar containing 500 cubic centimeters of tap water and a slice of banana. To this water was added a suspension prepared by macerating in distilled water five dengue-infected *A. ægypti* of lot 32. Similar suspensions, each made with five infective mosquitoes, were prepared daily and added to the water August 10, 11, 12, 13, 14, 15, and 16. August 17 the water in the jar was found to be slightly acid to litmus and was changed in the following manner. The larvæ were transferred to a clean jar containing 50 cubic centimeters of fresh tap water to which a suspension of five dengue-infected adult mosquitoes had been added. One hour later 400 cubic centimeters of clean water was added and the jar was placed in a screened cage. As some of the mosquitoes were already in the pupal stage, a few adults emerged on the following day, August 18. By August 20 only a few larvæ remained in the jar and the cage contained a considerable number of adult male and female mosquitoes. The females were separated and designated *A. ægypti* lot 62–B.

Test for infectivity.—August 26, 1929, or at least six days after they had emerged, thirty-three female mosquitoes of lot 62–B took blood from a susceptible volunteer (A.V. 36–V). Eight days later (September 3) some of the mosquitoes fed again on the same volunteer, and a third feeding with lot 62–B took place September 11, 1929. Dengue did not develop between September 11 and 26. The susceptibility of this volunteer was proved by another experiment in which twenty-eight *A. ægypti* of combined lots 44, 45, 46, and 47, which fed September 21, produced dengue after an incubation period of five days on September 26, 1929. Comment: In this experiment *A. ægypti* that had lived during their larval stage in 500 cubic centimeters of tap water containing banana, to which was added daily suspensions of five infected adult mosquitoes, failed to transmit dengue by feeding on a susceptible volunteer.

Experiment 2.—November 18, 1929. About two hundred recently hatched normal *A. ægypti* larvæ were placed in each of three fruit jars containing 250 cubic centimeters of distilled water, to which were added two macerated *A. ægypti* of lot 101 which had taken infective blood from A.V. 55–S during the first day of dengue. The infectivity of lot 101 was never determined, but the presence of dengue virus in the blood of A.V 55–S at the time of blood feeding was proved by lot 104, which later caused dengue in A.V. 56–K and A.V. 49–L. Subsequently, *A. ægypti* of lot 101 were added to the water in each jar as follows: November 19, two mosquitoes were added. November 20, two mosquitoes, were added; at 9 a. m., and at 12 noon a small slice of banana was placed in each jar. November 21, at 9 a. m., larvæ were transferred to bottles, each of which contained 30 cubic centimeters of distilled water and two mosquitoes of lot 101; and were left in the bottles for three hours, after which they were returned to the jars with 250 cubic centimeters of water. November 22, 23, and 24, two mosquitoes of lot 101 were added daily to each jar. November 25, at 9 a. m., larvæ were transferred to bottles containing 30 cubic centimeters of distilled water and two mosquitoes of lot 101, and were left in these bottles until the following day. November 26, at 10 a. m., the larvæ were returned to the jars and ten mosquitoes of lot 101, which had died during the previous day, were added to each jar; at noon thin slices of banana were added. November 27 and 28, two mosquitoes of lot 101 were added. November 29, at 7 a. m., the larvæ were transferred to bottles containing 30 cubic centimeters of distilled water and two mosquitoes of lot 101 and at noon they were returned to the jars containing 250 cubic centimeters of water and slices of banana. November 30 and December 1 and 2, two mosquitoes of lot 101 were added daily to each jar. December 3, at 8 a. m., larvæ were transferred to bottles containing 30 cubic centimeters of distilled water and two mosquitoes of lot 101 and were left until noon, after which they were returned to the jars as usual. December 4 and 5, two mosquitoes of lot 101 were added daily to each jar. December 6, larvæ were transferred to new jars with fresh water and banana. December 8, larvæ were transferred to clean fruit jars containing fresh water and banana and ten mosquitoes of lot 101 were added to each. The first pupæ appeared November 30, and as they developed they were transferred into bottles of distilled water and adults were allowed to emerge in a screened cage labeled "*A. ægypti* lot 127."

Test to determine infectivity of A. ægypti lot 127.—December 19, 1929, eighty-five mosquitoes of lot 127 took feedings of normal blood from Staff Sergeant Rhodes. After an interval of thirty-four days, one hundred ten mosquitoes of lot 127 fed on a volunteer (A.V. 76–M) at 8 a. m., January 22, 1930. Dengue did not develop during sixteen days observation. The susceptibility of this man (A.V. 76–M) to dengue was proved by the fact that sixty-one infective *A. ægypti* of lot 137, which fed on him February 1, 1930, produced dengue February 7, 1930. Comment: In this experiment *A. ægypti*, which, during the larval stage had developed in water repeatedly contaminated by additions of macerated potentially infected *A. ægypti*, failed to produce dengue in a susceptible human volunteer.

DISCUSSION

While the failure to infect *Aëdes ægypti* larvæ with dengue in these experiments seems to indicate that such infection may not be of importance in the natural spread of the virus, it is, of course, possible that larvæ might be infected under slightly different circumstances. So little is known concerning the biology of the virus of dengue, its viability in various mediums and under different conditions of temperature and hydrogen-ion concentration, or concerning the amounts of virus present in mosquitoes or other infective materials, that it will be necessary to investigate the question further before it can be stated that larvæ of *A. ægypti* are not susceptible to infection. For instance, it seems possible that after suspensions of infected mosquitoes were added to the water, the dilution of virus might have been so great that none of it was ingested by the larvæ; it is also possible that the virus was unable to survive in the water long enough to reach the larvæ in an infective condition. Under natural conditions *Aëdes* larvæ may be found developing in small collections of water in receptacles containing many different substances, which no doubt influence its hydrogen-ion reaction and its nutritive value, and it seems possible that further investigation might show that larvæ may be infected with dengue by the ingestion of virus contaminating the medium in which they develop.

SUMMARY AND CONCLUSIONS

1. Adult female *Aëdes ægypti*, developed from larvæ that had lived in water contaminated at intervals by the addition of small amounts of macerated infective adult mosquitoes, failed to transmit dengue to susceptible human individuals.
2. However, it was not possible to be certain that the dengue virus added to the water was ingested by these larvæ or that it remained alive long enough to cause infection even if it were ingested.
3. Further investigation may show that under different conditions mosquito larvæ may be infected with dengue virus.

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OBSERVATIONS ON THE POSSIBILITY OF TRANSMITTING DENGUE VIRUS FROM INFECTED TO NORMAL AËDES ÆGYPTI THROUGH CONTAMINATED FOOD OR WATER

As dengue virus escapes from the proboscis of infective female *Aëdes ægypti* while they are feeding on blood, it was considered possible that a similar escape of the virus might occur during the ingestion of other foods or water, and make it possible for normal mosquitoes feeding subsequently on the "contaminated" material to ingest the virus and become infected.

EXPERIMENTAL

In the following experiments an attempt was made to transmit dengue in this manner, by placing banana, papaya, sugar solutions, and water in the cages of infected mosquitoes for various periods of time and then transferring these materials to cages containing normal mosquitoes. After intervals of several days, the latter groups of mosquitoes were tested for infectivity by allowing them to feed on susceptible human volunteers.

Experiment 1.—August 1, 1929. Small glass containers for banana and papaya were prepared from sections of glass tubing, and cups for water were made by cutting off the lower portion of homeopathic vials. A fresh set of these containers, filled with water and fruit, respectively, was placed each day in a cage containing a large number of infective *A. ægypti*, lot 32. After use for twenty-four hours, the food and water containers were transferred to a cage containing two hundred normal *A. ægypti*. Large numbers of both groups of mosquitoes fed on the same materials. This procedure was kept up daily for several weeks, after which the normal-group mosquitoes were allowed to feed on normal human blood and reserved for later tests on human volunteers. Unfortunately, the mortality was so great among these mosquitoes that their infectivity could not be determined.

Experiment 2.—August 14, 1929. Papaya, banana, and water were transferred daily, as in experiment 1, from a cage containing infective *A. ægypti*, lot 30, to a cage containing one hundred normal *A. ægypti*, which were called lot 62–A. September 14, or thirty-one days after the beginning of the experiment, the six mosquitoes remaining in lot 62 were given an opportunity to feed on a susceptible volunteer (A.V. 46–L). Four of the mosquitoes took blood, but dengue did not develop during an observation period of twenty-three days. Comment: Because of the fact that only four of the original one hundred mosquitoes were available for test on the volunteer, these negative results were considered to be inconclusive.

Experiment 3.—November 18, 1929. A clean sterile Petri plate, containing approximately 15 cubic centimeters of 5 per cent sugar solution in tap water, was placed in a cage containing one hundred female *A. ægypti* selected from lot 101 and designated lot 101–A. The infectivity of these mosquitoes was indicated by the fact that other *A. ægypti*, lot 104, which fed at the same time as lot 101, transmitted dengue by feeding on human volunteers (A.V. 56–K and A.V. 49–L). After twenty-four hours (November 19) the sugar solution was transferred from the cage of *A. ægypti*, lot 101–A, to a cage containing one hundred normal *A. ægypti*, which were designated lot 121. Another clean dish of fresh sugar solution was given to lot 101–A. This routine transfer of used sugar solution from lot 101–A to lot 121 was continued daily for fifteen days, between November 20 and December 5, at which time only sixteen mosquitoes of lot 101–A remained alive.

TESTS FOR INFECTIVITY

Test (*a*).—November 28, 1929, 8.15 a. m. Susceptible volunteer A.V. 59–W was bitten by sixty-three of the sixty-seven mosquitoes remaining in lot 121 but dengue did not result.

Test (*b*).—December 5, 1929, 11.30 a. m. Forty-five mosquitoes of lot 121 again fed on the volunteer (A.V. 59–W) and again failed to cause dengue.

Test (*c*).—December 12, 1929. Thirty-one mosquitoes of lot 121 fed a third time on A.V. 59–W with similar negative results.

Test (*d*).—January 14, 1930, 1 p. m. Sixteen *A. ægypti* of lot 121 fed on another susceptible volunteer (A.V. 74–L), who also failed to develop dengue during an observation period of seventeen days.

Comment.—Normal *A. ægypti* of lot 121, which had fed daily for sixteen days on 15 cubic centimeter amounts of 5 per cent sugar solution that had been used by infective mosquitoes for twenty-four hours previously, were tested four times and failed to transmit dengue to man.

Experiment 4.—November 18, 1929. A sterile Petri plate containing fresh tap water and another plate containing a thin slice of ripe banana were placed in a cage containing one hundred eleven infective female *A. ægypti* selected form lot 101 and designated lot 101-B. Twenty-four hours later (November 19) the water and banana were replaced by fresh materials in new containers, and the previously contaminated water and food were transferred to a cage containing one hundred normal female *A. ægypti*, which were designated lot 122. Similar transfers of food and water were made daily for sixteen days, between November 19 and December 5, 1929.

TESTS FOR INFECTIVITY OF AËDES ÆGYPTI, LOT 122

Test (*a*).—November 28, 1929, 8.15 a. m. Sixty of the sixty-eight remaining mosquitoes of lot 122 fed on a susceptible volunteer (A.V. 59–W) but failed to produce dengue.

Test (*b*).—December 5, 1929, 11.30 a. m. Nineteen mosquitoes of lot 122 again fed on the same volunteer (A.V. 59–W) with similar negative results.

Test (*c*).—December 12, 1929, 9 a. m. Twenty-six mosquitoes of lot 122 fed on A.V. 59–W and failed to cause dengue.

Test (*d*).—January 14, 1930, 1 p. m. Six mosquitoes of lot 122 fed on a susceptible volunteer (A.V. 74–L) and failed to produce dengue.

Comment.—Normal *A. ægypti* of lot 122, fed daily for sixteen days on banana and water that had been used by infective mosquitoes for twenty-four hours previously, were tested four times and failed to transmit dengue to man.

Experiment 5.—March 10, 1930. One hundred thirty normal female *A. albopictus*, later called lot 182, were liberated in a cage with one hundred infective female *A. ægypti* of lot 168, which had fed on the blood of a dengue patient twenty-one days previously and had transmitted dengue to two human volunteers (A.V. 83–R and A.V. 87–R). All mosquitoes of both lots, No. 186 (*A. albopictus*) and No. 168 (*A. ægypti*), were inspected individually and identified before they were placed together in the cage. A small glass dish containing 15 cubic centimeters of a 5 per cent sugar solution was then added, in order that the normal *A. albopictus* might feed on the same solution as the infective *A. ægypti*. The food material was renewed daily during eleven days of the experiment.

TESTS TO DETERMINE INFECTIVITY OF AËDES ALBOPICTUS, LOT 182

Test (*a*).—March 22, 1930, 11 a. m. Sixty-eight of the remaining *A. albopictus*, lot 182, collected from the two mixed lots, fed on a susceptible volunteer (A.V. 92–C), but failed to produce dengue.

Test (*b*).—March 31, 1930, 10.30 a. m. Twenty-three *A. albopictus* of lot 182 again fed on the same volunteer (A.V. 92–C) and as dengue did not develop during an observation period of ten days, the susceptibility of this volunteer was tested April 10 at 12.30 p. m., by feeding on him seventy-three infective *A. ægypti* of lot 189. Two days five hours after this feeding dengue began in the afternoon of April 12. As this incubation period is shorter than any previously observed, it seems possible that the lot 182 *A. albopictus* might have caused the disease after a rather long incubation period of twelve days seven hours.

Comment.—From this experiment no final conclusion can be drawn concerning the possibility of transmitting dengue from infected *A. ægypti* to normal *A. albopictus* through contaminated food. However, the findings are sufficiently suggestive to warrant further investigation.

SUMMARY AND CONCLUSIONS

Five attempts were made to infect normal *Aëdes* by feeding them on food materials or water that had been used during the previous day by infective mosquitoes. In one experiment the mosquitoes died before they could be tested on a susceptible human volunteer; in one experiment only four mosquitoes remained for the test, which was negative; in two definitely negative experiments the mosquitoes failed to infect susceptible volunteers; and in one experiment in which normal *A. albopictus* were placed in the same cage with infected *A ægypti,* the results were suggestive but inconclusive. Thus, no positive evidence was obtained in these experiments to indicate that such transmission may occur. However, in the light of other experiments in which normal *Aëdes* were infected by the ingestion of macerated infected mosquitoes suspended in normal blood, it is believed possible that under favorable conditions mosquitoes may become infected with dengue virus in nature through the agency of contaminated food or water.

TRANSMISSION OF DENGUE VIRUS BY FEEDING NORMAL AËDES ÆGYPTI ON SUSPENSIONS OF INFECTED MOSQUITOES

In the following experiments dengue virus was transmitted directly from infective to normal *Aëdes ægypti* without an intermediate passage through a warm-blooded host, by macerating and suspending the infective mosquitoes in normal blood and feeding this suspension to the normal mosquitoes. This was made possible by the development of a satisfactory method (1) for feeding mosquitoes artificially on blood and other materials, which is described below.

EXPERIMENTAL

Technic.—The fresh, clean skin of a young, normal, exsanguinated guinea pig or chicken, freed of subcutaneous fat and hair or feathers, was clamped tightly over the shallow depression on the under surface of the lid of an ordinary staining dish (Plate 2, fig. 1) by means of a wire frame held in place by rubber bands or by sealing with paraffin. The air was removed from the cell with a Luer syringe and replaced by a mixture of citrated or defibrinated normal blood[4] and macerated dengue-infected *Aëdes ægypti*. Sufficient material was injected to cause a distinct bulging of the skin, after which the needle hole was sealed with melted paraffin. The mixture was kept at approximately 37° C. by placing the apparatus over a glass dish half filled with water heated to 55° C. The entire apparatus was then inserted into a cage containing normal laboratory-bred *A. ægypti*. The mosquitoes were at once attracted to the skin, and in a short time most of them became engorged with the blood mixture (Plate 2, fig. 2). This technic was carried out on eight different occasions with successful results in four.

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[4] The blood used was obtained from volunteers during the observation period preliminary to dengue experimentation and in one instance from a normal guinea pig. The human blood used was proved to be free from the virus at the time it was taken, by subsequent observation of the volunteers for eight days, and by producing dengue fever in these individuals, a few days after the end of the observation period, with known infected mosquitoes.

Experiment 1.—August 20, 1929. Eighty infective *Aëdes ægypti* of lot 32, which had fed thirty-four days previously on an experimental case of dengue (A.V. 21–M) during the first day of the disease, and had been proved infected by transmitting dengue fever to A.V. 28–P and A.V. 29–J, were macerated in a mortar containing sterile sand, 1 cubic centimeter of 0.85 per cent salt solution (NaCl), and 4 cubic centimeters of normal human blood and fed to twenty-two normal *A. ægypti*, which were designated lot 44.

August 20, 1929. Twenty-five infective *A. ægypti* of lot 35, which had fed sixteen days previously on an experimental case of dengue (A.V. 28–P), during the first day of the disease, and had been proved infective by transmitting dengue fever to A.V. 31–D, were macerated with sand and 4 cubic centimeters of citrated human blood; and fed to twenty-two normal *A. ægypti*, which were designated lot 45.

August 22, 1929. Forty *A. ægypti* of lot 40, which had fed seventeen days previously on an experimental case (A.V. 20–B), at the beginning of the second day of dengue fever, but had not subsequently been proved infective, were macerated and suspended in 3 cubic centimeters of guinea-pig blood, and fed to seven normal *A. ægypti*, which were designated lot 46.

August 23, 1929. Thirty infective *A. ægypti* from lot 40, and thirty from lot 41, which had fed eighteen and seventeen days previously on experimental cases A.V. 20–B and A.V. 23–B, were macerated and suspended in 5 cubic centimeters of normal human blood and fed to one hundred one normal *A. ægypti*, which became lot 47.

September 5, 1929. Thirteen to sixteen days after the mosquitoes of lots 44, 45, 46, and 47 had ingested blood from the dengue patients, they were tested for infectivity by allowing forty-seven of them to feed on a susceptible volunteer (A.V. 39–M); who, after an incubation period of six days, developed a typical unmodified case of dengue fever, which continued for seven days.

September 21, 1929. Twenty-nine to thirty-two days following the infective feedings by lots 44, 45, 46, and 47, twenty-eight fed on A.V. 36–V, who five days later developed a typical and rather severe case of dengue fever. The diagnosis in this case was further proved by the passage of the virus from this volunteer through *A. ægypti* lot 71 to A.V. 48–H and A.V. 55–S and subsequently through several human subjects and different lots of mosquitoes.

Conclusions.—Normal *A. ægypti*, which had fed on blood containing a suspension of macerated dengue-infected mosquitoes in the proportions of 1.6, 0.625, 1.33, and 1.20 mosquitoes to 0.1 cubic centimeter of fluid, became infected as was proved by the transmission of the disease to two susceptible American volunteers.

Experiment 2.—September 23, 1929. Five *A. ægypti*, of lot 48, which had fed twenty-seven days previously on A.V. 29–J during the first day of dengue fever, but which had not been tested, were macerated and suspended in 8.5 cubic centimeters of normal human blood, and fed to one

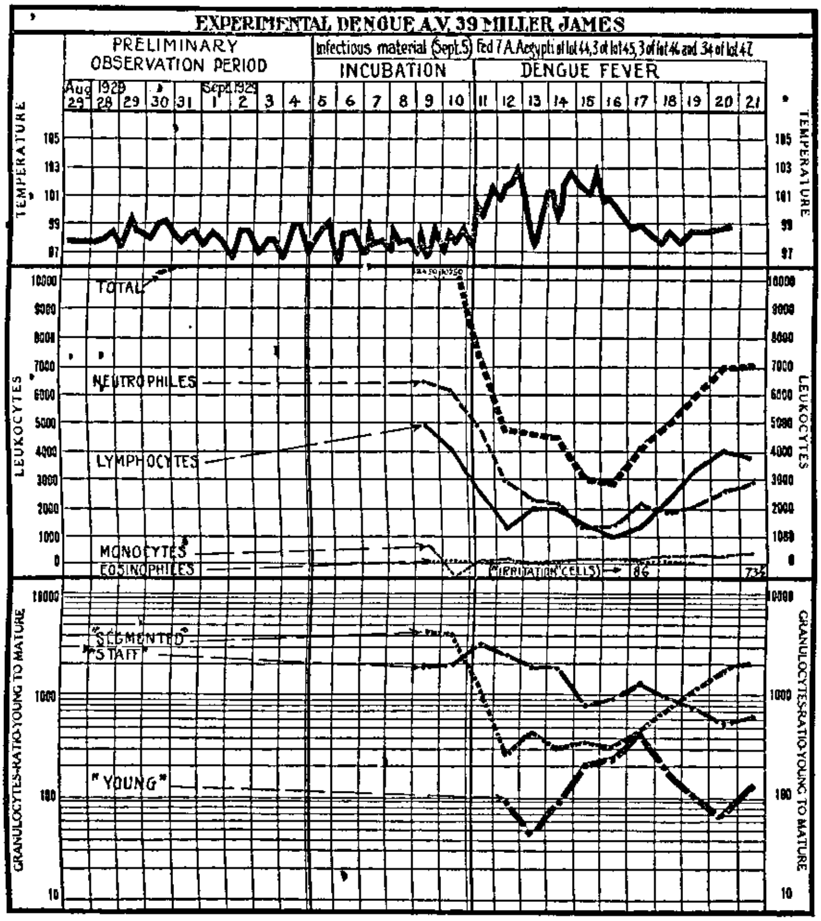

FIG. 12. Experimental dengue in American volunteer 39.

hundred forty-eight normal *A. ægypti*, which were designated lot 67 and tested for infectivity as follows: October 7, 1929, fourteen days following feeding on this mosquito suspension, six *A. ægypti* of lot 67 fed on a volunteer (A.V. 49–L), but no infection followed. Eight days later (October 15), or twenty-two days after the original feeding, forty-seven mosquitoes of lot 67 again fed on the same volunteer. Dengue did not follow during a prolonged observation period, and the volunteer was subsequently proved susceptible by the feeding of twenty-one infective *A. ægypti* of lot 104, November 16, 1929, which produced dengue November 21, 1929.

Conclusions.—The feeding of normal *A. ægypti* on a dilute suspension of presumably dengue-infected *A. ægypti* (0.058 mosquito per 0.1 cubic centimeter of blood) did not result in their infection as was indicated by the fact that small and large groups fed on a susceptible volunteer failed to transmit dengue.

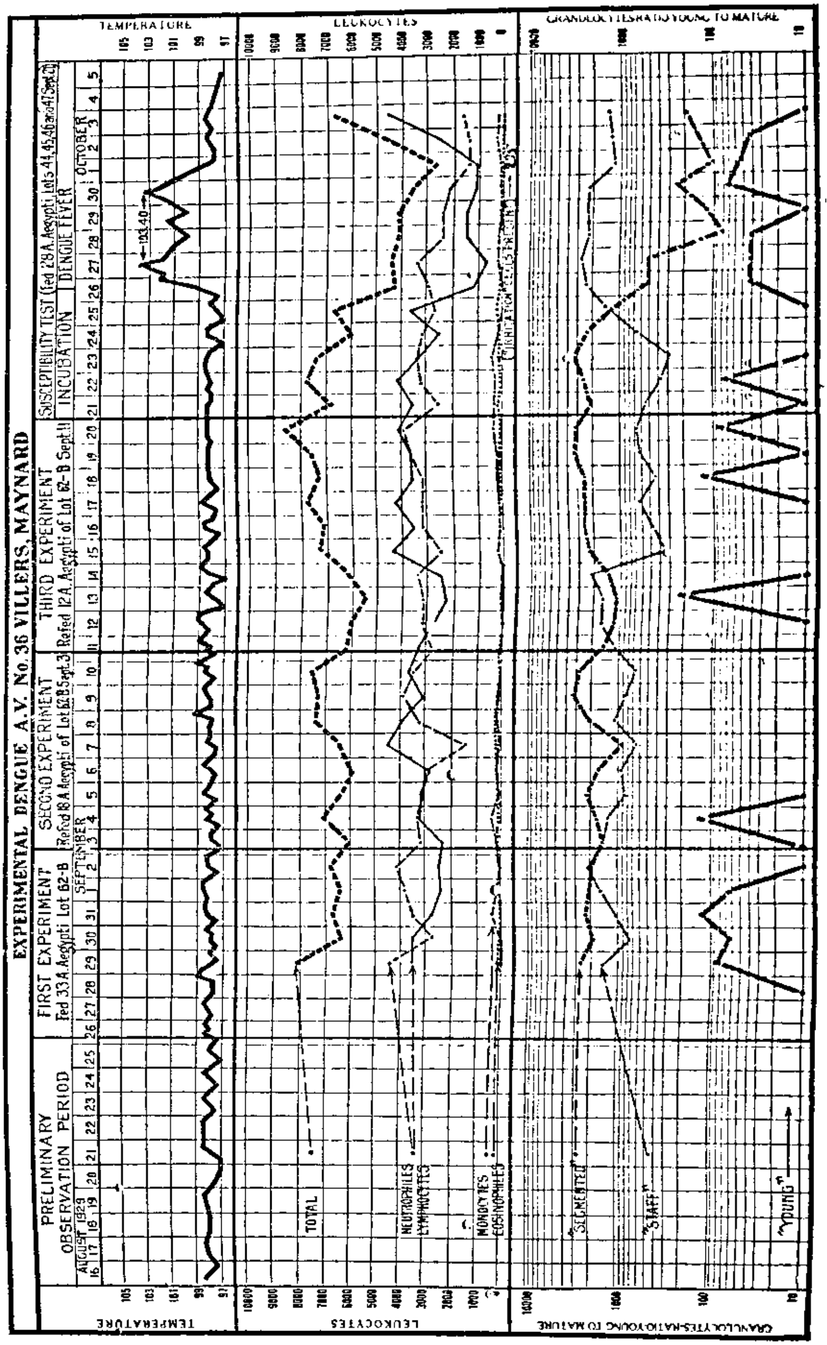

FIG. 13. Experimental dengue in American volunteer 36.

Experiment 3.—September 23, 1929. Fifty *A. ægypti* of lot 48, which had fed twenty-seven days previously on an experimental dengue case (A.V. 29–J) during the first day of fever, but had not been tested, were ground in a mortar containing a little sand and a few drops of hormone broth. After adding sufficient hormone broth to make a total volume of 10 cubic centimeters, an attempt was made to feed this material to normal *A. ægypti.* As the mosquitoes failed to feed, 5 cubic centimeters of the suspension was removed and replaced by 5 cubic centimeters of normal blood. Fifty-eight normal *A. ægypti* fed on this mixture and were designated lot 68. October 23, or thirty days later, seventeen mosquitoes of lot 68 were tested for infectivity by feeding on a volunteer (A.V. 49–L). Dengue did not follow during an observation period extending to November 16. The volunteer was subsequently proved susceptible by the infective feeding of *A. ægypti,* lot 104, November 16, 1929, which resulted in dengue November 21, 1929.

Conclusions.—Normal *A. ægypti* fed on a mixture of equal parts hormone broth and normal blood, which contained a suspension of presumably dengue-infected *A. ægypti* in the proportion of 0.25 mosquito to 0.1 cubic centimeter of fluid, failed to transmit dengue to man.

Experiment 4.—October 30, 1929. Eighty-one *A. ægypti;* namely, six of lot 52, eight of lot 54, seven of lot 59, one of lot 60, two of lot 61, five of lot 71, ten of lot 72, thirty-two of lot 76 and ten of lot 79, which had fed on the blood of experimental dengue patients from twenty-one to sixty days previously, were ground in a mortar containing 1 cubic centimeter of 0.85 per cent salt solution and 1 cubic centimeter of normal human blood. Thirty-three normal *A. ægypti* that fed on this mixture were designated lot 96.

November 11, 1929. Twelve days later the mosquitoes of lot 96 were tested for infectivity by feeding thirty of them on a volunteer (A.V. 57–E). This man developed dengue fever, which began five days later.

Conclusions.—Normal *A. ægypti* fed on blood containing dengue-infected mosquitoes (4.05 per 0.1 cubic centimeter of fluid) were infected as was proved by their transmission of dengue to a volunteer (A.V. 57–E) and the subsequent transfer of the infection from this man through lot 114 *A. ægypti* to another volunteer (A.V. 66–W).

Experiment 5.—March 5, 1930. One hundred *A. ægypti* of lot 165, which twenty-five days previously had fed on an experimental case (A.V. 76–M), during the first day of dengue fever, and had subsequently transmitted dengue to A.V. 86–B, were ground in a mortar containing powdered pyrex glass, 1 cubic centimeter of 0.85 per cent salt solution (NaCl) and 2 cubic centimeters of normal defibrinated human blood and fed to fifty-two normal *A. ægypti,* which were designated lot 177 and tested for infectivity as follows:

March 22, 1930. Seventeen days after feeding on the mosquito suspension sixteen mosquitoes of lot 177 were fed on a susceptible volunteer (A.V. 93–R). Dengue did not follow and the volunteer's susceptibility was proved subsequently by means of lot 183 *A. albopictus.*

Conclusions.—Normal *A. ægypti* fed on a suspension of infective mosquitoes (3.33 mosquitoes per 0.1 cubic centimeter of blood) failed to transmit dengue to man.

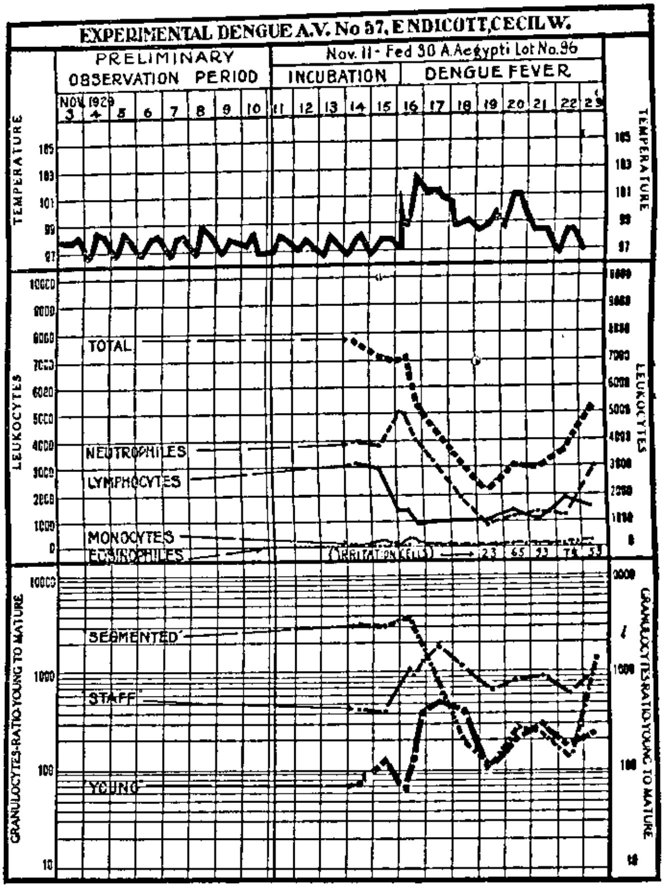

FIG. 14. Experimental dengue in American volunteer 57.

Experiment 6.—March 5 and 7, 1930. On these dates two groups, each composed of one hundred *A. ægypti* of lot 168, which had fed sixteen and eighteen days previously on an experimental dengue case (A.V. 78–O) during the first day of fever, and had transmitted dengue to two volunteers (A.V. 83–R and A.V. 87–R), were ground in a mortar containing powdered pyrex glass, 1 cubic centimeter of 0.85 per cent salt solution, and 2 cubic centimeters of normal human blood. This material was then fed to sixty-five and sixty-three *A. ægypti*, respectively, which were designated lots 178 and 181, and later tested for infectivity as follows:

March 14, 1930. Nine and seven days, respectively, following the feeding of lots 178 and 181 on the mosquito suspension, forty-four and thirty-two of them fed on A.V. 88–B, but failed to cause dengue. However, when twenty-eight and nineteen of these mosquitoes were fed again, March

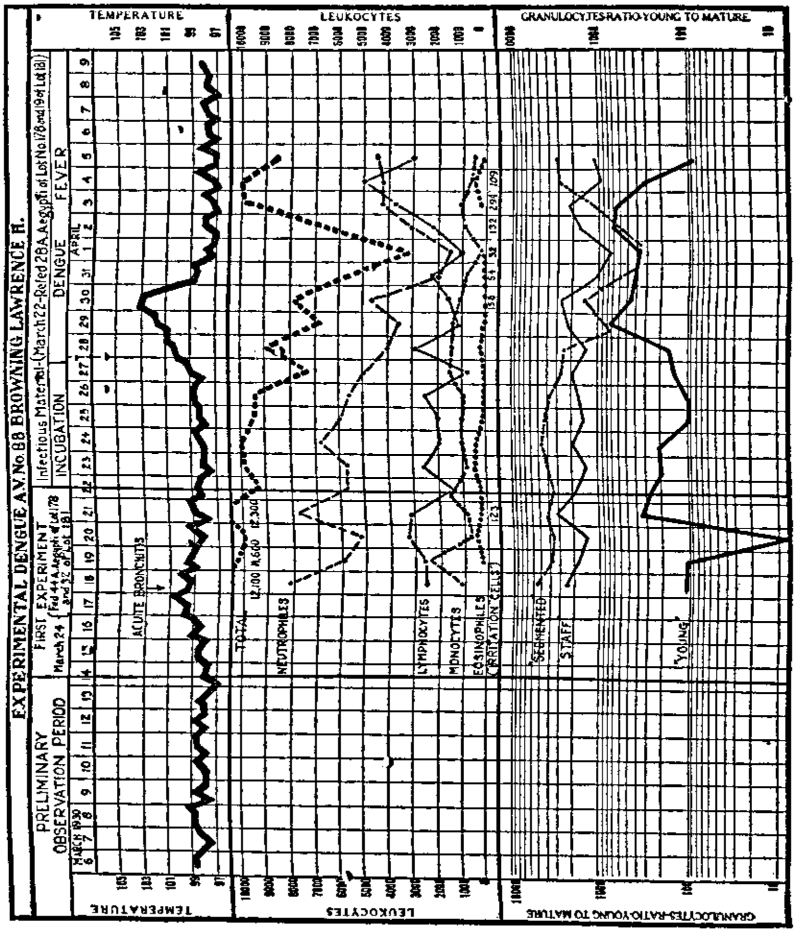

FIG. 15. Experimental dengue in American volunteer 88.

22, on the same volunteer a mild but typical case of dengue developed, after an incubation period of five days.

Comments.—(1) As dengue was not transmitted by lots 178 and 181 nine and seven days after they had ingested the suspension but was carried by these mosquitoes after the intervals had become seventeen and fifteen days, it appears that the former interval of time was too brief for the development of the virus within the mosquitoes.

(2) Normal *A. ægypti* were infected by feeding on a suspension of macerated dengue-infected *A. ægypti* containing 3.33 mosquitoes per 0.1 cubic centimeter of fluid.

Experiment 7.—September 16 and 17, 1929. On these dates attempts were made to effect a second serial transfer of the virus of dengue from mosquito to mosquito. September 16, eighteen *A. ægypti* of lots 44, 45, 46, and 47, which had previously been infected by feeding on suspensions of mosquitoes (see experiment 1, Table 6), were macerated in a mortar containing sand and 4 cubic centimeters of defibrinated normal human blood, and fed to thirteen normal *A. ægypti.* September 17, twenty-four additional mosquitoes of the same lots (44, 45, 46, and 47) were macerated and suspended in 5 cubic centimeters of normal human blood and fed to eleven normal *A. ægypti.* The mosquitoes were combined, designated lot 63, and tested for infectivity as follows:

October 2, 1929. Fifteen and sixteen days, respectively, after the feeding on the infective suspension, seven *A. ægypti* of lot 63, fed on a susceptible volunteer (A.V. 44–F); but dengue did not result. The susceptibility of this man was proved subsequently with lot 52 *Aëdes ægypti.*

Conclusion.—The attempt to transfer dengue virus in series by feeding normal *A. ægypti* on a normal blood suspension of mosquitoes (0.45 and 0.48 mosquito per 0.1 cubic centimeter), which themselves had been infected by feeding on a suspension of infective *A. ægypti,* was unsuccessful.

Experiment 8.—September 23, 1929. A second attempt was made on this date to transfer dengue virus in series from mosquito to mosquito. Thirty *A. ægypti* of the combined lots 44, 45, 46, and 47 (see experiment 1) were macerated and suspended in 1 cubic centimeter of normal human blood, and 5 cubic centimeters of 0.85 per cent salt solution. One hundred thirty-five normal *A. ægypti,* which fed on this suspension, were designated lot 69. September 28, or five days after this feeding, ninety-five mosquitoes of lot 69 were fed on a volunteer (A.V. 47–D); as dengue did not develop during the following eight days, fifty mosquitoes of the same lot were again fed on A.V. 47–D October 7. After an incubation period of five days a mild attack of dengue developed. The diagnosis was further confirmed by transfer of the virus from A.V. 47–D through *A. ægypti* lot 86 to two other volunteers, A.V. 51–S and A.V. 50–C, both of whom developed typical dengue.

November 4, 1929. Forty-two days after the infective feeding, ten mosquitoes of lot 69 were again tested on a volunteer (A.V. 56–K). As dengue did not develop during the following twelve days, the volunteer was tested for susceptibility November 16, 1929, by feeding *A. ægypti* of lot 104. A typical, moderately severe attack of dengue developed November 23, 1929.

Comments.—(1) Apparently infections occurred only in certain individual mosquitoes of the groups that fed on the suspension representing a dilution of 0.5 mosquito per 0.1 cubic centimeter of saline and blood, and these infected mosquitoes had died before the second test. The mosquitoes used in preparing this suspension had themselves been infected by feeding on a suspension of dengue mosquitoes; therefore, this experiment represented a second serial transfer of dengue virus from infected to normal mosquitoes.

(2) This experiment also demonstrates that the mosquitoes are not infective five days after feeding on other mosquitoes but are able to transmit the infection after an interval of fourteen days.

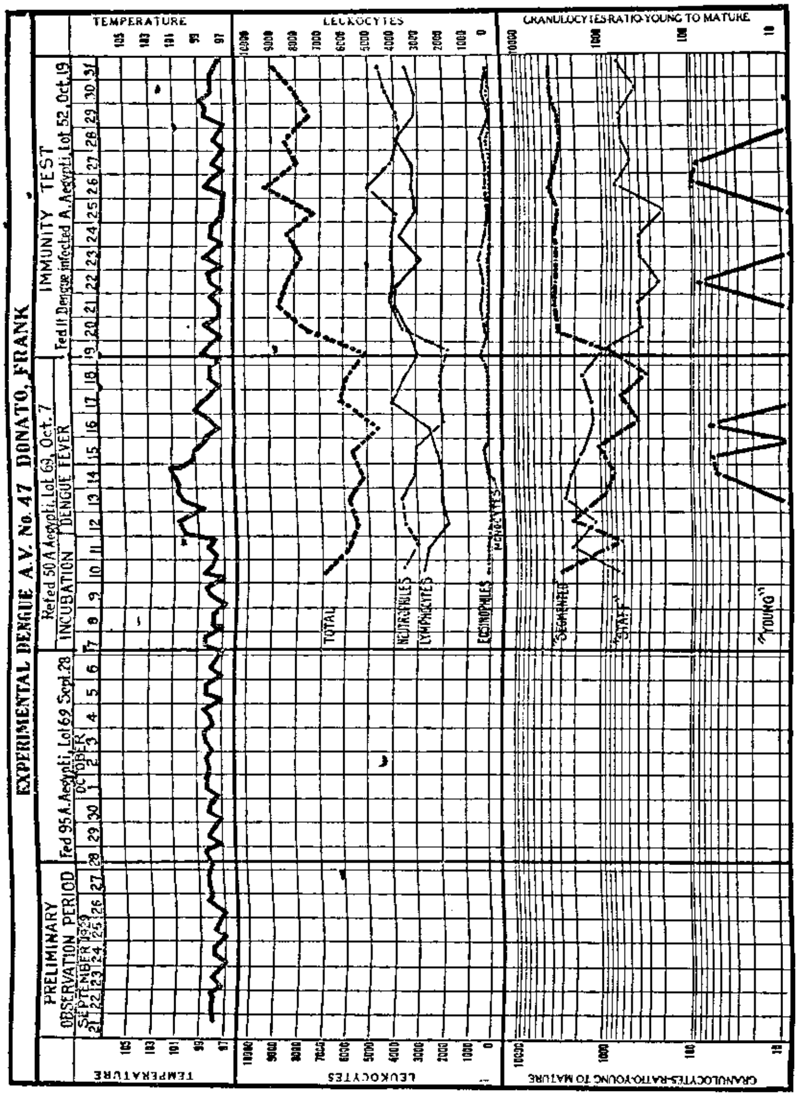

FIG. 16. Experimental dengue in American volunteer 47.

DISCUSSION

The results obtained in the experimental attempts to pass the virus of dengue directly from mosquito to mosquito seem to indicate that the degree of concentration of the virus in the mosquito-blood mixture used to infect normal *Aëdes* is an important factor. In the mixtures used in the successful experiments, the concentrations were about 0.5, 1.21, 3.33, and 4.05 mosquitoes, respectively, per 0.1 cubic centimeter of fluid, while in the unsuccessful tests the mixtures contained about 0.058, 0.25, 0.45, and 3.33 mosquitoes to 0.1 cubic centimeter of fluid. As it has been shown that a single *A. ægypti,* during a normal feeding, ingests about 0.00253 gram, or about 0.002 cubic centimeter, of blood, it may be presumed that during the successful experiments the normal mosquitoes each ingested about 0.012, 0.031, 0.083, and 0.102, respectively, of one infected mosquito, while in the unsuccessful experiments each normal mosquito ingested 0.0015, 0.006, 0.011, and 0.083 *A. ægypti.* The most-dilute infective suspension used successfully was that employed in experiment 8, in which each normal mosquito ingested material representing about 0.01 of an infected mosquito; and at least 7 per cent of the lot fed on this material were unable to transmit dengue.

The possibility of decreasing the virulence of dengue virus by serial mosquito passages is of interest. As was noted in experiments 1, 4, and 6, all of which dealt with primary transfers of virus from mosquito to mosquito, one of the volunteers developed a severe case of dengue, two had average cases, and one person had a mild infection. The one case of dengue caused by mosquitoes infected by a second serial transfer of virus was milder than usual. Thus, while the virulence of dengue virus is apparently not modified by the first passage from infected to normal *A. ægypti,* there is a possibility that serial passages through mosquitoes might result in decreased virulence of the virus.

It should be noted that in experiments 6 and 8 the mosquitoes which fed on volunteers five and seven days after the ingestion of virus failed to transmit dengue, while the same lots did infect volunteers when tested on a later date. Thus, it would appear that the virus from infected mosquitoes, as that obtained from warm-blooded animals, must pass through a period of development or multiplication in *Aëdes* before the mosquito can infect man through feeding.

SUMMARY AND CONCLUSIONS

1. The virus of dengue has been transferred from infective to normal mosquitoes by feeding normal *Aëdes ægypti* on infected mosquitoes macerated and suspended in normal blood.

2. Successful passage of dengue virus in this manner was obtained with a mixture containing not less than five infective mosquitoes per cubic centimeter of blood.

3. The virus apparently retained its virulence for man after the initial passages from mosquito to mosquito. There is a possibility that the virulence of dengue virus might be decreased after serial passages through mosquitoes.

4. A period of more than seven days was required before normal mosquitoes that had ingested dengue virus from mosquito suspensions could transmit dengue to man.

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ATTEMPTS TO DEMONSTRATE THE VIRUS OF DENGUE BY MICROSCOPIC EXAMINATION

For many years workers have searched for the organism responsible for dengue fever. McLaughlin(1) in 1886 published an account of his investigations concerning the etiology of dengue and reported having found a spherical micrococcus which was one-twentieth to one-thirtieth the diameter of an erythrocyte, both in the fresh and incubated blood of dengue cases and in nutrient gelatin cultures made with the blood. Graham(2) in 1903, working in Syria, described an organism seen within red blood cells which, except for the presence of pigment, resembled *Plasmodium malariæ*. He reported having found these bodies in the stomachs of *Culex fatigans* up to the fifth day following their feeding on dengue patients, and also the finding of spores of the organism in the salivary glands of the mosquito. Later workers, including Carpenter and Sutton (Isthmus of Panama, 1903),(3) Guiteras and Cartaya (Cuba, 1905),(4) Report of the Bureau of Government Laboratories (Manila, P. I., 1900),(5) Agramonte (Havana, Cuba, 1906),(6) Kiewiet de Jonge and de Haan (Java, 1905),(7) Stitt (Philippine Islands, 1906),(8) and Ashburn and Craig (Philippine Islands, 1907),(9) were unable to confirm the findings of McLaughlin and Graham. Furthermore, they were unable to consistently demonstrate in the blood any organism that they could logically consider of etiological significance.

Later investigators were concerned with attempts to demonstrate in dengue-infected materials a spirochæte or *Leptospira* similar to the *Leptospira* found by Noguchi(10) in cases diagnosed as yellow fever. Couvy,(11) (1921) after direct examination of the blood of dengue patients, reported having found a short slender spirochæte; while Siler, Hall, and Hitchens(12) observed a spirochæte-like form in the blood of several dengue patients, which they considered had nothing to do with the etiology of dengue as was indicated by their careful control work with blood of normal individuals. Carbo-Noboa in 1924(13) reported finding spirochætes in the serum, citrated blood, and cerebrospinal fluid of dengue patients, examined microscopically with dark-field illumination and after the use of Levaditi's stain. Knowles and Des Gupta (14) came to the conclusion that spiral structures found in the blood of dengue patients in Calcutta, which were at first thought to be *Leptospira*, were in reality artefacts. De Faria (15) found a *Leptospira* in dengue patients in Brazil; and Vervoort (16) observed "*Leptospira pyrogones*" in a denguelike fever in India. However, Chandler and Rice (17) from experiments on man and animals obtained no evidence to indicate that the causative agent of dengue was a *Leptospira*, an observation which agrees with the results of Knowles and Des Gupta, (14) Holt, (18) Siler, Hall, and Hitchens, (12) Duval and Harris, (19) and the authors of this report. When it was shown that yellow fever is probably not due to a *Leptospira*, further search for such an organism in dengue was discouraged; and the attention of investigators has been directed into other channels.

Duval and Harris, (19) using Noguchi's (10) medium, found "globoid" bodies, measuring from 0.1 to 0.3 micron, in cultures made with dengue blood. Sellards and Siler, (20) in a study of histological sections from normal and dengue-infected *Aëdes ægypti*, found masses of *Rickettsia* in the hind gut of infected mosquitoes, but none in a control group of normal mosquitoes. The *Rickettsia* were not seen in the salivary glands or in any tissue outside of the alimentary tract. Moutoussis (21) also found *Rickettsia* and inclusion bodies in *Stegomyia* infected with dengue virus. However, Schule (22) writes that during examinations of *A. ægypti* fed on dengue blood and of others fed on normal blood, he regularly found in both groups of mosquitoes organisms identical with those described by Sellards and Siler.

It is apparent that much time has been spent in attempts to demonstrate visually an organism responsible for dengue fever; and that at various times bacteria, Protozoa, *Leptospira*, and *Rickettsia* have been suggested as of possible etiological significance. However, none of these organisms has been definitely incriminated. On the other hand, considerable evidence has accumulated to indicate that the virus, in the blood of patients and in infected mosquitoes, is probably too small to be seen by any means now available.

EXPERIMENTAL

Microscopic examination of blood from dengue patients.—Blood specimens obtained daily from two cases of experimentally induced dengue (A.V. 1–H and A.V. 2–M), during the incubation period and throughout the disease, and specimens from two others (A.V. 3–A and A.V. 4–Y), collected on each day of the disease, were prepared in the following manner and examined microscopically: (*a*) Dark-field preparations; (*b*) films stained by Wright's, Giemsa's, and Mann's methods and with flagella stains. The results were negative. A number of blood preparations were treated with 95 per cent alcohol, tannic acid, saturated solution of ammonium sulphate, formalin and copper sulphate in various dilutions, and then stained with Giemsa's stain, Wright's stain, gentian violet and other dyes, but none of the preparations contained anything recognizable as the virus of dengue.

Attempts to render the virus visible by precipitation and concentration.—Microscopic examinations were also made of infective blood and serum which had been mixed with convalescent serum and centrifugalized in the hope that agglutination or precipitation of the virus might result in masses large enough to be seen. The results of these experiments were also negative.

Microscopic examination of inoculated culture media.—Both aërobic and anaërobic cultures were attempted by inoculating blood containing dengue virus from experimental dengue patients into Noguchi's medium, N. N. N. medium, Locke-egg-serum medium for amœba, Hibler's brain medium, Francis's cystine agar, beef infusion broth enriched with equal parts of hydrocele fluid or ascitic fluid, banana broth, hormone broth (heart), brain, spleen, and bone-marrow broth. No evidence of dengue virus was obtained by microscopic examination of the incubated materials in these tubes.

Microscopic examination of infective mosquitoes.—Repeated attempts to identify the virus of dengue in saline suspensions of *A. ægypti*, were unsuccessful. Smear preparations of the salivary glands of individual dengue-infected mosquitoes, examined microscopically with dark-field illumination, and after staining by Giemsa's and Wright's methods, failed to show any form that could not be demonstrated in control normal mosquitoes. The salivary glands of dengue-infected *Aëdes* were removed in groups of ten infected *A. ægypti* each, macerated in a small amount of salt solution, and immediately examined microscopically, using the "dark-field" and various staining methods, including Giemsa's and Wright's. No forms were seen in these preparations that could not also be found in materials from normal mosquitoes.

Microscopic examinations of serum contaminated by infective mosquitoes.—As the virus of dengue escapes from the proboscis of infected mosquitoes during feeding, an attempt was made to collect a concentrated suspension of virus by feeding 100 infective *A. ægypti*, lot 72, on a small amount of guinea-pig serum (1 cubic centimeter) that had been diluted with an equal amount of 0.9 per cent salt solution and placed in a shallow cell beneath a clean piece of skin which had just been removed from a young chicken. As a control on this procedure, approximately one hundred fifty normal *A. ægypti* were allowed to feed on another mixture of serum and salt solution similarly arranged. After the mosquitoes had fed, the serum was removed from beneath the skin and centrifugalized at 2,000 revolutions per minute for thirty minutes. Direct microscopic examination was made of the wet sediment, using both the ordinary and dark-field illumination. Dry films were stained with hæmatoxylin and eosin, and by Wright's, Giemsa's, Gram's and Hiss's (capsule) methods. Minute coccoid and rod-shaped forms were observed in all of the preparations, from both the control and the test serum, but these forms appeared to be much more numerous in the latter.

CONCLUSIONS

1. The virus of dengue was not identified by microscopic examinations of the following materials: (*a*) Blood from cases of experimentally induced dengue, (*b*) materials from culture media inoculated with the virus, (*c*) crushed *A. ægypti* infected with virus, (*d*) the salivary glands of infective *A. ægypti*, or (*e*) clear guinea-pig serum on which one hundred infective *A. ægypti* had fed.

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CULTIVATION EXPERIMENTS WITH THE VIRUS OF DENGUE

Many attempts have been made to cultivate the causative agent of dengue; but, so far, no one has furnished convincing proof that this virus is able to multiply outside of the living tissues of its hosts. McLaughlin(1) in 1886, using the limited methods of that period, studied cultures inoculated with the blood of dengue patients; but his preparations were contaminated with skin cocci and the results are of historical interest only. In 1907 Ashburn and Craig(2) approached the problem, "with high hopes that by applying modern cultivation methods we might be able to isolate and cultivate the organism causing the disease. In view of the success attained by Novy and others in the cultivation of trypanosomes, and by Rodgers in cultivating the Leishman-Donovan body (*Herpetomonas donovani*), we were especially hopeful that by applying similar methods in this disease, we might be able to secure growths of any protozoön which might be present." These investigators made cultures of dengue blood collected in citrate solutions at various periods of the disease and incubated them at different temperatures; cultures were also made by adding 10 cubic centimeters of blood to 250 cubic centimeters of bouillon in flasks. As a result of their experiments it was concluded that no organism was found constantly enough in the cultures to warrant regarding it as the cause of dengue. In 1914 Lavinder and Francis(3) also failed to demonstrate any growth of virus in cultures prepared by inoculating dengue blood into glucose broth, and incubating the tubes at 37° C. and at room temperature for several days. Chandler and Rice(4) in 1923, using methods which had proven successful with *Leptospira*, were unable to observe any growth of dengue virus. In 1924 Duval and Harris,(5) using Noguchi's medium, reported having grown minute filterable anaërobic globoid bodies, which after inoculation into guinea pigs gave rise to reactions similar to those that they had observed following injections of blood from dengue cases. While these workers considered that the globoid bodies might have had an etiological relationship to dengue they recognized that further proof was required before this could be established. Siler, Hall, and Hitchens(6) in 1926 attempted to grow the organism of dengue in various media, including (*a*) the rabbit kidney ascitic fluid of Smith and Noguchi, (*b*) the semisolid *Leptospira* medium of Noguchi, and (*c*) the peptone-acid-phosphoricum of Vervoort. These media, inoculated with blood specimens from forty-five dengue patients and incubated at 37° C. or at room temperature for intervals as long as one month, failed to show any recognizable gross or microscopic evidence of growth of the virus of dengue. Schule(7) during 1927 also made cultures with infective dengue blood inoculated into various types of media. He isolated a minute, filter-passing, "Gram-doubtful" coccobacillus in the cultures from one case. However, injection of this organism into susceptible human volunteers failed to cause dengue or to produce any immunity to the disease; and he, therefore, considered his cultivation experiments to be negative. Copanaris(8) and Manoussakis,(9) during the recent (1927–1928) epidemic in Athens, also failed to cultivate the virus of dengue.

In spite of the formidable array of negative experiments reported by previous workers, cultivation of dengue virus was again attempted by the authors, using different types of media and new methods for determining the presence or absence of virus in cultures.

PRESENT INVESTIGATION

The cultivation experiments may be divided into two main groups. In the first group various culture media, including Noguchi's serum medium, Boeck and Drbohlav's amœba medium, Hibler's brain medium, brain agar, N. N. N. medium, Francis's cystine agar, dextrose infusion broth, and mosquito broth, were inoculated with potentially infected materials from dengue patients, incubated aërobically and anaërobically at 37° C. and at room temperature, and observed both macroscopically and microscopically for evidence of growth. In the second group of experiments, it had been planned to test the cultures for the presence of virus, even though there was no visual evidence of growth, by passing the incubated material through diatomaceous filters followed by injection of the filtrates into susceptible volunteers. This procedure was not carried out because of variable results obtained in filtration experiments with known infected materials. However, the development of a satisfactory method(10) for feeding *Aëdes ægypti* artificially on infected materials, placed in a shallow cell beneath a piece of guinea-pig or chicken skin, made it possible to test the cultures for virus in this manner.

1. CULTURE MEDIA INOCULATED WITH INFECTIVE MATERIALS AND LATER EXAMINED MACROSCOPICALLY AND MICROSCOPICALLY FOR EVIDENCE OF THE GROWTH OF DENGUE VIRUS

Experiment 1.—January 2 to 12, 1929. Fresh specimens of urine and blood were collected early in the incubation period, and on the first, second, and fourth days of dengue fever, from an experimental case (A.V. 1–H). Normal *Aëdes ægypti* fed on this man on the second day of the fever and called lot 3 were later proved to be infected with the virus. After inoculating 0.1 cubic centimeter amounts of the material into the various media mentioned above, sets of both aërobic and partially anaërobic cultures (under paraffin oil) were incubated at 37° C. and at room temperature for several weeks. All of the tubes were observed daily, and microscopic examinations of dark-field preparations and of films stained by Wright's and Gram's methods were made at frequent intervals. A few miscellaneous bacterial contaminants grew in some of the tubes inoculated with urine, but none showed any evidence of dengue virus.

Experiment 2.—January 2 to 12, 1929. Fresh specimens of urine and blood collected early in the incubation period and on the second, third, fourth, and fifth days of dengue fever, from an experimental case (A.V. 2–M), were inoculated into culture media and observed as in experiment 1. The blood collected from this man on the second day of fever was infective as was proved later by passage of the virus through *A. ægypti* (lot 2). No evidence of growth of the virus was observed in these tubes.

Experiment 3.—February 25, 1929. Cultures were prepared similarly with blood immediately after its collection in a small amount of sodium citrate from an experimental case (A.V. 3–A) on the morning of the second day of dengue fever. The infectivity of this blood was indicated by the fact that *A. ægypti* fed on this man at the same time (lot 9) later transmitted dengue. Additional aërobic and anaërobic cultures were prepared by inoculating flasks containing 100 cubic centimeters of dextrose infusion broth with 0.2 cubic centimeter amounts of the citrated blood. After three weeks of negative observations the inoculated materials were discarded.

Experiment 4.—February 27, 1929. Similar tests were made with fresh citrated blood collected in the forty-ninth hour of dengue fever from an experimental case (A.V. 4–Y). The fact that normal *A. ægypti* designated lot 10, which fed on this man at the same time, were later proved infected indicates that the blood inoculated in the media also contained dengue virus. The cultural results were negative.

Experiment 5.—April 14, 1929. Fresh blood was collected in citrate from an experimental case (A.V., 6–R) during the first day of dengue; and 0.5 cubic centimeter amounts were inoculated into tubes of the following media: (*a*) Noguchi's medium, (*b*) N. N. N. medium, (*c*) cystine agar, and (*d*) beef-infusion broth. Similar inoculations were made (*e*) into flasks containing 100 cubic centimeters each of 1 per cent dextrose broth. It may be assumed that the blood contained virus, since normal *A. ægypti* fed on the patient, at the time of its collection, were later used to transmit dengue (lot 12). However, no evidence of development of the virus was observed in the cultures.

Experiment 6.—May 15, 1929. Fresh blood was collected in citrate, from a dengue case (A.V. 9–D) during the second day of the fever. The infectivity of the blood was proved by feeding normal *A. ægypti* (lot 19), which were later used to transmit dengue. The blood was inoculated into culture media as in experiment 5 and was incubated and observed as usual. The results were negative.

Experiment 7.—April 29, 1929. Fresh citrated blood was collected from a dengue case (A.V. 10–H) early on the second day of fever. Normal *A. ægypti* fed on the patient at the same time were later proved infected (lot 17). The dengue-infected blood was inoculated into media in the usual manner, but evidence of growth of dengue virus was not observed during a period of one month.

Experiment 8.—July 16, 1929. *Attempts to cultivate dengue virus in a mosquito-broth medium.* Because of the readiness with which normal *A. ægypti* may be infected with dengue virus, and the apparent ease with which the virus continues to develop in these mosquitoes after infection, it was considered possible that a culture medium containing mosquito protein might favor growth of the virus in vitro. Therefore, a large number of normal *A. ægypti* were killed, weighed, and macerated in a sterile mortar with sufficient Locke's solution to make a 2 per cent suspension, and the mosquito suspension was then sterilized by filtration. Because of the limited supply of material, this medium was used in 0.9 cubic centimeter amounts in long slender tubes prepared from narrow glass tubing. After inoculation with 0.1 cubic centimeter amounts of the infected serum or blood, the tubes were incubated at 37° C.; and at two- to four-day intervals 0.1 cubic centimeter amounts were transferred to fresh tubes of the medium. The blood used in this experiment was collected in sodium citrate from a case (A.V. 22–M) early on the second day of dengue; and was proved infective at the time by *A. ægypti* (lot 30) and *A. albopictus* (lot 29). The inoculated tubes of media were examined daily during a period of two weeks, but there was no evidence of growth of virus.

Experiment 9.—September 6, 1929. Fresh citrated infective blood drawn from a dengue case (A.V. 34–S) on the second day of fever was inoculated over the surface of seven thin slices each of (*a*) boiled banana and (*b*) raw banana. Examination of the cultures for a period of two weeks failed to show any evidence of the growth of dengue virus.

2. CULTURES INOCULATED WITH INFECTIVE MATERIALS AND LATER TESTED FOR GROWTH OF VIRUS BY FEEDING TO NORMAL AËDES ÆGYPTI

In the following experiments an attempt was made to cultivate the dengue virus in a number of different types of special media. Material from the inoculated tubes was transferred serially at different intervals of time so that the final tubes contained such great dilutions of the original inoculum as to decrease the possibility of obtaining positive results by preservation of small amounts of virus rather than by its multiplication. As it had been shown that normal *A. ægypti* might be infected by feeding on dengue virus suspended in normal blood and placed in a shallow feeding cell, advantage was taken of this method for determining the presence or absence of virulent dengue virus in cultures. Therefore, after inoculation of the medium with dengue-infected materials and incubation of the transferred culture materials, this was fed to normal mosquitoes, which were later tested for infectivity by feeding on susceptible volunteers. The following culture media were used: (*a*) Galloway's relapsing-fever spirochæte medium;(11) (*b*) Locke-egg-serum medium for amœbæ; (*c*) N. N. N. medium; (*d*) hydrocele fluid; (*e*) hormone (heart) broth; (*f*) spleen broth; (*g*) brain broth; (*h*) bone-marrow broth; (*i*) banana broth; and (*j*) lymph-gland broth containing bone. These media were prepared as follows:

(*a*) *Galloway's medium for relapsing-fever spirochætes.*—Small amounts of whole-egg emulsion were placed in test tubes, and heated in a water bath until coagulated; 5 cubic centimeters of rabbit serum, which had been heated for half an hour at 58° C. and then diluted 1 to 5 with physiologic salt solution, liquid petrolatum, and 1 drop of normal blood were then added to each tube.

(*b*) *Boeck and Drbohlav's Locke-egg-serum medium for amœbæ.*(12)—Sodium chloride 9 grams, calcium chloride 0.24 gram, potassium chloride 0.42 gram, sodium bicarbonate 0.20 gram, and dextrose 2.50 grams were dissolved in 1,000 cubic centimeters of distilled water, filtered, and autoclaved at 15 pounds pressure for fifteen minutes. Four whole eggs for each 50 cubic centimeters of solution were added, the medium tubed, and coagulated in slants. Each slant was then covered in whole or part by inactivated horse serum diluted with eight parts of Locke's solution. The medium was then incubated at 37° C. to test sterility.

(*c*) *"N. N. N." medium.*—Fourteen grams of agar and 6 grams of salt (NaCl) were dissolved in 900 cubic centimeters of distilled water by autoclaving at 15 pounds for one hour; 33⅓ per cent fresh defibrinated rabbit blood was added and the medium tubed in slants. The tubes were sealed with paraffined stoppers.

(*d*) In experiment 5 the medium was hydrocele fluid combined with equal parts of veal-infusion broth.

(*e*) *Hormone (heart) broth. Modification of Huntoon's method.*—Five hundred grams of finely minced beef heart, 500 cubic centimeters of distilled water, 10 grams of peptone, 5 grams of salt (NaCl), and one whole egg well beaten were mixed together, heated to 70° C., filtered through a wire strainer, titrated, and adjusted to p_H 7.6; heated in "Arnold" one hour and after standing for several hours the clear supernatant fluid was syphoned, tubed, and sterilized by the fractional method.

(*f*) *Spleen broth.*—Five hundred grams of finely ground bovine spleen were placed in 1,000 cubic centimeters of distilled water, heated in the

"Arnold" for one hour, and strained through cotton. One per cent peptone and 0.5 per cent salt (NaCl) were added, the reaction adjusted to p_H 7.6, tubed, and autoclaved at 15 pounds pressure for one hour.

(*g*) *Brain broth.*—Five hundred grams of minced calf brain were placed in 500 cubic centimeters of distilled water, sterilized in "Arnold" for one hour, 1 per cent peptone and 0.2 per cent salt (NaCl) were added and the reaction was adjusted to p_H 7.6; it was filtered through cheese cloth and cotton until the medium was clear, and sterilized at 15 pounds pressure for thirty minutes.

(*h*) *Bone-marrow broth.*—Five hundred grams of macerated fresh bovine bone-marrow were placed in 1,000 cubic centimeters of distilled water, containing 10 grams of peptone, 5 grams of salt (NaCl), and a whole egg well beaten. The mixture was then heated to 70° C., filtered through a wire sieve and adjusted to p_H 7.6, heated in the "Arnold" for one hour, allowed to stand over night, and the clear supernatant fluid was then pipetted, tubed in 10 cubic centimeter amounts, and sterilized fractionally at 15 pounds pressure.

(*i*) *Banana broth.*—Three hundred grams of fresh ripe banana were macerated, 200 cubic centimeters of distilled water added, heated, and cleared by centrifugalization. After the reaction was adjusted to p_H 7.4, the medium was tubed and sterilized fractionally.

(*j*) *Lymph-gland broth containing bone.*—Ten grams of peptone (Fairchild's) and 5 grams of sodium chloride in 100 cubic centimeters of distilled water were heated to boiling to dissolve, were cooled, and 500 grams of finely macerated normal bovine lymph glands were added. After storage at 18° C. for twenty-four hours, the medium was autoclaved, the reaction adjusted to p_H 7.6 and filtered through paper for clearing, tubed in 9.9 cubic centimeter amounts in test tubes, which contained a small piece of fresh, clean guinea-pig bone. The medium was then covered with liquid petrolatum and autoclaved.

EXPERIMENTS

Experiment 1.—September 11, 1929. Fresh citrated infective blood withdrawn from an experimental case of dengue (A.V. 38–H) whose symptoms began in the afternoon September 10, was inoculated in 0.1 cubic centimeter amounts into each of the following media: (*a*) Galloway's medium; (*b*) Locke-egg-serum medium; (*c*) equal parts of hydrocele fluid and beef-infusion broth with and without a covering of petrolatum; and (*d*) N. N. N. medium. In each medium the transplants were made as follows: Ten tubes, each containing about 5 cubic centimeters of fluid, with the exception of the N. N. N. medium, were placed in a test-tube rack. Tube 1 was inoculated with 0.1 cubic centimeter of undiluted dengue blood. After incubation for one day 0.1 cubic centimeter was transferred to tube 2, and so on daily until the tenth tube was reached. Transfers were also made in series from tube 1, at two-day intervals to tubes 3, 5, 7, and 9; at three-day intervals to tubes 4, 7, and 10; and at four- and five-day intervals to tubes 5 and 10. Thus, various incubation periods were represented by the material in the last two tubes.

Culture materials fed to mosquitoes.—September 21, 1929, or ten days later, the contents of the last two tubes (tubes 9 and 10) of each medium, were mixed with normal human citrated blood collected from A.V. 42–H and fed to mosquitoes as follows: Cultures in media "a" and "b" were fed to one hundred twenty normal *A. ægypti* designated lot 64; cultures in medium "c" were fed to twenty-four normal *A. ægypti*, which became lot 65, and cultures in medium "d" were fed to twenty-nine normal *A. ægypti* designated lot 66.

TEST FOR VIRUS IN MOSQUITOES FED ON CULTURES

Test A.—October 7, 10.30 a. m., or sixteen days after feeding on the various culture-blood mixtures, eighty-two *A. ægypti* of lot 64, twenty-four of lot 65, and nineteen of lot 66 fed on a susceptible volunteer (A.V. 48–H). Dengue did not develop during twenty days observation.

Test B.—October 15, 9.30 a. m., or twenty-four days after feeding on the mixture of cultures and blood, thirty-five *Aëdes* of lot 64, twelve of lot 65, eleven of lot 66, and two of lot 74 again fed on the same volunteer (A.V. 48–H) and again failed to cause dengue during a period of twelve days observation.

Test C.—October 19, 10 a. m., or twenty-eight days after feeding on the culture-blood mixture, twenty-six *A. ægypti* of lot 64 fed on a susceptible volunteer (A.V. 51–S). Dengue did not develop in twelve days observation, and this lot was discarded November 1.

Test D.—October 19, 10 a. m., six *A. ægypti* of lot 65 and ten of lot 66 fed on a susceptible volunteer (A.V. 52–N). Since no symptoms of dengue occurred during the following twelve days this experiment was also considered negative, and therefore this volunteer was used on the evening of October 31 for another experiment, designed to test the infectivity of a different lot of *A. ægypti* (lot 99). However, November 1 at 10.30 a. m., or twenty-four hours following the feeding of lot 99 *A. ægypti* and thirteen days after the last feeding of lots 65 and 66, A.V. 52–N developed symptoms of dengue. Eighty-eight normal *A. ægypti* were fed at this time and designated lot 105, but were not tested. Comment: The fact that this volunteer (A.V. 52–N) was absent from the ward the night of October 27, just five days before he developed dengue makes it impossible to be sure of the source of his infection. However, considering the fact that thirteen days had elapsed since the feeding of six and ten *A. ægypti*, respectively, of lots 65 and 66; and the fact that about twice as many mosquitoes of these same lots had failed to infect A.V. 48–H who was bitten by them October 7 and again October 15, it seems possible that these mosquitoes of lots 65 and 66 were not responsible for the infection.

Experiment 2.—September 21, 1929. Infected blood was collected from an experimental case of dengue (A.V. 37–M) early on the second day of fever, and was inoculated in 0.1 cubic centimeter amounts into tubes containing 9.9 cubic centimeters each of hormone broth (heart), spleen broth, brain broth, and banana broth. From these first tubes (I), which contained the original blood in dilutions of 1 to 100, immediate transfers of 0.1 cubic centimeter amounts were made to a second series of tubes (II), resulting in dilutions of 1 to 10,000; from the second 0.1 cubic centimeter was carried to a third series (III), resulting in dilutions of 1 to 1 million; and from the latter tubes 0.1 cubic centimeter amounts were transferred to a fourth series (IV), resulting in dilutions of 1 to 1 billion. After incubation at 37° C. transfers were made from tubes I, II, and III at one-, two-, and four-day intervals for eight days, as follows: From the first tube in series I, 1 cubic centimeter amounts were transferred each day until September 28, giving a dilution of 1 to 1 billion in the final tube; from the first tube in series II, 0.1 cubic centimeter amounts were transferred every second day until September 27, resulting in a final dilution of 1 to 10 billion; from the first tube in series III, 0.01 cubic centimeter was transferred on September 25, giving a final dilution of 1 to 1 billion; while tube 1 in series IV, in which the blood was already diluted 1 to 1 billion, was incubated for eight days without transfer.

Culture materials fed to mosquitoes.—September 29, after the material in the final tubes of each series had been incubated for one, two, four, and eight days, respectively, representative portions of the culture materials were pooled, mixed with equal parts of citrated normal guinea-pig blood, and fed to normal *A. ægypti.* The final cultures in hormone broth and banana broth were pooled, mixed with blood in a feeding cell, and placed in a cage containing one hundred recently emerged normal mosquitoes, only eight of which fed on the mixture. Similar unsatisfactory results were obtained with the culture material in the brain broth and spleen broth, on which only ten of one hundred mosquitoes fed. A final attempt was made to feed additional mosquitoes on the following day, but only a few ingested the culture-blood mixture. The thirty-six mosquitoes fed on these cultures were combined and designated lot 80.

Test for virus in the mosquitoes.—Twelve days later five *A. ægypti* of lot 80 fed on a newly admitted volunteer (A.V. 50–C). Dengue fever did not result in this case.

Conclusions.—The small numbers of *A. ægypti* that fed on these cultures and the smaller number left for use in testing the infectivity of the lot, rendered the negative results of this experiment inconclusive.

Experiment 3.—September 27, 1929. Three series of tubes were prepared containing, respectively, (*a*) brain broth, (*b*) spleen broth, and (*c*) bone-marrow broth. The first tube in each series was inoculated with 1 cubic centimeter of citrated infected blood drawn thirty minutes earlier from a patient (A.V. 42–H), who had developed symptoms of dengue fever twenty-four hours previously. Thereafter, transfers were made daily to fresh tubes of media resulting in the following dilutions of the original blood: Brain-broth and spleen-broth mediums.—Tube 1 (1–10), 2 (1–100), 3 (1–2,000), 4 (1–40,000), 5 (1–800,000), 6 (1–160 millon), 7 (1–320 million), 8 (1–64 billion). Bone-marrow broth.—Tube 1 (1–10), 2 (1–100), 3 (1–1,000), 4 (1–20,000), 5 (1–200,000), 6 (1–2 million), 7 (1–20 million), 8 (1–200 million).

Culture materials fed to mosquitoes.—October 5, a mixture was prepared by adding 5 cubic centimeters of defibrinated normal human blood to 1 cubic centimeter each of the bone-marrow broth (1–200 million), brain broth (1–6.4 billion), and spleen broth (1–6.4 billion) culture materials. After placing this culture-blood mixture in a feeding cell, seven normal *A. ægypti* fed and were designated lot 75. After an additional two days incubation another mixture was prepared from the cultures as follows: One cubic centimeter bone-marrow broth (1–200 million), 2 cubic centimeters bone-marrow broth (1–20 million), 1 cubic centimeter spleen broth (1–64 billion), and 1 cubic centimeter brain broth (1–64 billion) to which was added 5 cubic centimeters of defibrinated normal human blood. Forty-eight normal *A. ægypti*, which fed on this mixture, were added to the seven mosquitoes remaining in lot 75.

Test for virus in mosquitoes.—October 21, 10 a. m., fourteen *A. ægypti* of lot 75 and one of lot 80 fed on a susceptible volunteer (A.V. 50–C). Dengue did not develop.

Conclusion.—There was no indication that the virus of dengue grew in these cultures.

Experiment 4.—December 4, 1929. Blood containing virus was obtained from an experimental case (A.V. 62–H) during the beginning of dengue. Normal *A. albopictus* which fed at the same time, designated lot 128, later produced dengue in two monkeys (*M. philippinensis* 30 and 31) and in A.V. 69–K. This infective blood was immediately inoculated in 0.1 cubic centimeter amounts into tubes containing approximately 6 cubic centimeters each of the following media: (*a*) Equal parts of hydrocele fluid and veal-infusion broth (*b*) the same medium covered with paraffin oil, and (*c*) Boeck and Drborhlav's amœba medium. After incubation at 37° C., serial transplants of 0.1 cubic centimeter amounts were made December 5, 7, 9, 11, and 13. December 17, the final cultures in each medium were mixed with equal parts of guinea-pig blood, making a total of about 15 cubic centimeters of fluid, a part of which was then fed to normal *A. ægypti.*

TESTS OF THE MOSQUITOES FOR INFECTIVITY

Test A.—The normal *A. ægypti* fed on the hydrocele-fluid medium were called lot 140. Fifty mosquitoes of this lot later fed on a susceptible volunteer (A.V. 67–McC) at 9.30 a. m., January 13, 1930, but failed to cause dengue. This lot was retested at 10 a. m., January 24, 1930, when thirty-four mosquitoes again fed on a susceptible volunteer (A.V. 77–E) and again failed to produce dengue. The lot 140 mosquitoes were considered uninfected and were discarded February 12, 1930.

Test B.—Normal A. *ægypti* fed on the hydrocele-fluid medium incubated under paraffin, and designated lot 141, were tested along with lot 140. Thirty of these mosquitoes fed on A.V. 67–McC January 13, and twenty-one fed on A.V. 78–O January 24, 1930. As dengue did not develop in either of these susceptible volunteers the lot 141 mosquitoes were considered uninfected, and were discarded February 12, 1930.

Test C.—Normal *A. ægypti* fed on the cultures in the amœba medium were designated lot 142 and tested on a susceptible volunteer (A.V. 71–W). Ninety mosquitoes fed on this man January 13, 1930; and seventy again fed January 27, 1930. As the results were negative these mosquitoes were discarded February 13, 1930.

Conclusion.—There was no evidence indicating growth of the dengue virus in these cultures.

Experiment 5.—December 4, 1929, 10.45 a. m. Blood was collected from an experimental case (A.V. 62–H) early on the second day of dengue and 0.1-cubic-centimeter amounts were inoculated into tubes containing 5 cubic centimeter amounts of the following media: (*a*) Brain broth, (*b*) brain broth covered with paraffin oil, (*c*) spleen broth, (*d*) spleen broth covered with paraffin oil, (*e*) hormone broth (heart), (*f*) hormone broth covered with paraffin oil, (*g*) bone-marrow broth, (*h*) bone-marrow broth covered with paraffin oil. Each culture was incubated at 37° C., and at two-day intervals 0.1 cubic centimeter amounts were transferred serially to new tubes of media.

Culture material fed to mosquitoes.—December 23, 1929, each final culture was mixed with equal parts of normal guinea-pig blood and fed artificially to normal *A. ægypti* which were designated as follows: One hundred thirty-eight mosquitoes fed on the brain broth (*a*), lot 147; one hundred thirty mosquitoes fed on the brain broth (*b*), lot 148; one hundred three fed on spleen broth (*c*), lot 149; one hundred forty fed on spleen broth (*d*), lot 150; sixty fed on hormone broth (*e*), lot 151; one hundred fifty fed on hormone broth (*f*), lot 152; one hundred thirty-eight fed on bone-marrow broth (*g*), lot 153; and ninety-six mosquitoes fed on bone-marrow broth (*h*), lot 154.

Test for virus in mosquitoes.—These various lots of *A. ægypti* were tested for dengue virus as follows:

Test A.—January 13, 1930, ninety-three, ninety-eight, eighteen, and one hundred two mosquitoes of lots 147, 149, 151, and 153, respectively, fed on a susceptible volunteer (A.V. 71–W) and failed to cause dengue.

Test B.—January 27, 1930, eighty-five, seventy-five, twelve, and one hundred sixteen mosquitoes of the same lots again fed on A.V. 71–W with negative results.

Test C.—January 13, 1930, eighty-one, one hundred twelve, ninety-five, and eighty-seven *A. ægypti* of lots 148, 150, 152, and 154, respectively, were fed on a susceptible volunteer (A.V. 68–J) and failed to cause dengue.

Conclusion.—There was no evidence of the growth of dengue virus in any of these cultures.

Experiment 6.—December 16, 1929. Defibrinated blood was collected from a dengue patient (A.V. 66–W) during the first day of fever and inoculated in 0.1 cubic centimeter amounts into tubes containing 9.9 cubic centimeters each of lymph-gland broth. Transfers were made serially of 0.1 cubic centimeter amounts at two-day intervals for twenty-eight days. January 15, 1930, the final culture was mixed with an equal amount of normal guinea-pig blood and fed artificially to one hundred fifty-five normal *A. ægypti*, called lot 155. This lot was tested at 10.30 a. m., February 15, 1930, when twelve of the mosquitoes fed on a susceptible volunteer (A.V. 79–H). As the volunteer did not develop dengue from this biting, the mosquitoes were considered to be free from virus.

Comment.—No evidence was obtained to indicate that dengue virus was cultivated in the media used.

DISCUSSION

The results of these cultivation experiments agree with the negative observations of other workers and indicate that dengue virus, as it exists in infected blood, is probably incapable of multiplication in vitro in the culture media used. However, as the etiological agent of dengue has not yet been recognized morphologically and its presence is detected only by demonstrating its ability to cause typical infection in man, the evidence at hand is hardly sufficient to prove that dengue virus failed to reproduce in these experiments. For example, it is at least possible that the virus added to the initial tubes of media might have developed and been carried by transfer to the final tube, but that due to some change during cultivation it had become incapable of infecting the normal mosquitoes by which it was ingested. It is also conceivable that the virus might have developed in virulent form in all of the cultures, but that due to slow growth or to its great dilution in the final tubes of media, it was not present in sufficient concentration to cause infection of the mosquitoes. However, the latter possibility is considered to be unlikely. It has been shown by the authors that during an average complete feeding normal *A. ægypti* ingest about 2.5 milligrams of blood; and that this amount of blood from a dengue patient, or a similar amount of a mixture containing five infective *A. ægypti,* suspended in 1 cubic centimeter of normal blood, is sufficient to infect normal mosquitoes. Since relatively large numbers of mosquitoes took full feedings from the cultures, it appears that the chances favored their infection, had the virus been present even in small amounts. For example, one hundred *A. ægypti,* each ingesting 2.5 milligrams from 5 cubic centimeters of equal parts of normal blood and culture material spread in a thin layer with an area of 3 by 4 inches, would result in the testing of one hundred different samples of 2.5 milligrams each, representing a total of 250 milligrams of the culture-blood mixture. Therefore, in spite of the unexplained result obtained in test D, experiment 1, it seems likely that the virus of dengue failed to multiply in the media used, as is indicated by the negative results obtained in the other transmission experiments.

CONCLUSIONS

1. In these cultivation experiments no macroscopic or microscopic evidence was observed indicating growth of the virus of dengue in vitro.

2. The negative results of the tests on susceptible volunteers to determine the possible infection of *A. ægypti* that had fed on inoculated media indicate that dengue virus failed to grow in the culture media used.

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THE VIABILITY OF DENGUE VIRUS

Information concerning the viability of dengue virus under different conditions is of considerable importance; however, because of the rather difficult and tedious technical procedures necessary for the detection of dengue virus, such information is limited and is largely incidental to various other experimental observations considered to be of more immediate importance. The available information indicates that dengue virus is able to exist for relatively long periods in the bodies of certain infected mosquitoes, for shorter periods in infected human beings and lower animals, and for very short periods outside the living bodies of these hosts.

Viability of dengue virus in Aëdes mosquitoes.—It has been shown by Siler, Hall, and Hitchens,(1) Blanc and Caminopetros,(2) and the present authors that dengue virus remains alive in infected *A. ægypti* for periods of at least seventy-five, one hundred seventy-four, and seventy days, respectively; and the authors have recently shown that the virus lives for at least fifty-four days in *A. albopictus*. Apparently, the living tissues of these mosquitoes furnish ideal conditions for the continued existence of dengue virus, which probably lives until the death of the insect. While there is very little information available concerning the duration of life of the virus in infected mosquitoes after their death, it has been shown by filtration experiments with suspensions of killed *A. ægypti* that the virus remained alive, at room temperature, for at least two hours. However, attemps to demonstrate virus in the dried tissues of *A. ægypti* after longer periods were not successful, as is shown in the following experiments.

Experiment 1.—March 3, 1930. One hundred infective *A. ægypti*, including sixty-nine of lot 162, nine of lot 135, seven of lot 137, five of lot 138, and ten of lot 139, were killed, ground in a mortar, desiccated over calcium chloride, and stored in a vacuum in a sealed glass tube, kept in the refrigerator.

After four days storage (March 7) the mass of dry mosquito tissue was suspended in 3 cubic centimeters of a mixture consisting of 1 cubic centimeter of physiologic salt solution and 2 cubic centimeters of defibrinated normal human blood. One hundred twenty-four normal *A. ægypti* fed on this mosquito suspension and were designated lot 179. March 22 and 24, 1930, sixteen of these lot 179 mosquitoes were tested for infectivity by feeding on a susceptible volunteer (A.V. 91–K). Dengue did not result.

Experiment 2.—March 3, 1930, one hundred infective *A. ægypti* of lot 164 were killed, dried, and stored in a manner similar to that followed in experiment 1. Four days later the material was mixed with blood and fed to sixty-seven normal *A. ægypti* which were designated lot 180. Twelve of these mosquitoes fed on a susceptible volunteer (A.V. 91–K) March 24, 1930, but failed to cause dengue.

Comment.—While these two negative experiments fail to prove that the virus had died after being kept for four days in the dried tissues of dead *A. ægypti,* it seems probable that this was the case.

Viability of dengue virus in blood.—After virulent virus has been introduced into susceptible human beings it remains alive throughout the incubation period of from four to ten or more days, and during at least the first three, and probably more, days of the infection. This is no doubt true also of infections in susceptible lower animals, particularly monkeys.

Thus, the life of the virus in man may be estimated at from seven to fifteen days, with the possibility that very minute amounts of virulent virus or larger amounts of attenuated virus may exist for much longer periods. It is apparent that the small amount of virus introduced by a mosquito during the infective biting multiplies more or less rapidly, until within four to ten days it is present in sufficient amount to cause symptoms of dengue fever. During the day preceding the fever the concentration of virus in the blood may or may not be great enough to cause infection in normal mosquitoes which ingest full feedings of about 0.025 gram of the blood.

Mosquitoes fed on the first and second days of the disease practically always become infected, indicating that at this time the concentration of infective virus is at its height. On the third day of fever there is probably a diminution in virulent virus, as mosquitoes may or may not be infected by feeding at this time; while on subsequent days mosquitoes can rarely be infected by feeding on the blood of dengue patients. However, from the reports of various investigators who have produced dengue by inoculating volunteers with large amounts of blood collected from patients at different periods, it appears possible that while diminished in amount virulent virus may be present throughout the fever. As there is no method available for the recognition of attenuated virus, it is, of course, also possible that during infection the virus may not be killed but may continue to live and multiply in an attenuated form, which might be transmissible to insects and might even revert to the infective state at a later period. While this possibility is purely speculative there is no available experimental evidence to indicate that the virus of dengue is killed in man or that a deferred carrier state might not occur in this host.

VIABILITY OF VIRUS IN INFECTIVE BLOOD AFTER ITS COLLECTION FROM DENGUE PATIENTS

Cleland, Bradley, and McDonald(3) (1919) produced dengue in volunteers by inoculations of infective blood which had been kept at refrigerator or room temperatures for periods of 48, 172, and 99 hours, respectively. Koizumi, Yamaguchi, and Tonomura(4) (1918) reported that in two instances dengue virus in infective blood, which was diluted with three and four volumes of saline, was infective after standing for five hours at room temperature, while similar materials kept in the ice chest gave negative results when injected into volunteers. Blanc and Caminopetros(5) (1929) found that the addition of one-fifteenth volume of bile to dengue blood rendered the virus non-infective within five minutes, while smaller amounts caused a decrease in virulence. Manoussakis(6) (1928) preserved virus for six days by collecting 25 cubic centimeters of infective blood during the first twenty-four hours of dengue, and mixing this with 200 cubic centimeters of normal saline, which was then sealed and stored in an incubator. Six days later the subcutaneous inoculation of 6 cubic centimeters of the supernatant fluid caused dengue. He also observed that the infective power of dengue virus in serum was destroyed by heating to 50° C. for half an hour, while exposure to 40° C. for a similar period did not interfere with its virulence. Virus was not destroyed by exposure to sunlight for half an hour, or by storage for seven days at 37° C., or at room temperature for eight days. Blanc, Caminopetros, and Manoussakis(7) (1928) announced that dengue virus in serum, stored in sterile flasks at room temperature, remained infective for a period up to two months. Although the observations of various workers concerning the viability of dengue virus after removal from the living tissues of its hosts differ considerably, it is apparent that under favorable conditions the virus may live for several days. Although somewhat resistant to unfavorable conditions, the many attempts made to cultivate dengue virus have been unsuccessful; and as yet no one has been able to prove that it can multiply in vitro.

The recent announcement by Sawyer, Lloyd, and Kitchen(8) (1929), of a method by which yellow-fever virus may be preserved for at least one hundred fifty-four days by freezing the infective blood of monkeys immediately after collection, drying it in a vacuum while frozen, and storing the dried blood in sterile glass containers kept in the refrigerator, led to the following experiments in which an effort was made to preserve dengue virus in a similar manner.

ATTEMPTS TO PRESERVE DENGUE VIRUS IN FROZEN DRIED BLOOD

Blood was collected from experimental dengue-fever patients during the first day of the disease, and 0.5 to 1 cubic centimeter amounts were immediately placed in sterile Petri plates, evaporating dishes, or small test tubes, and frozen at once, either in an ice-brine mixture or in solid carbon dioxide. The frozen blood was then placed in a desiccator surrounded by ice and brine, and was dried over calcium chloride until the following day when it was sealed in pyrex glass tubes. After storage at 18° C. for different intervals of time, portions of the samples of dried blood were suspended in physiologic salt solution and tested for the presence of infective virus by subcutaneous inoculation into susceptible volunteers. As indicated in Table 1, no evidence was obtained to show that any of these preparations contained infective dengue virus after storage for periods of four, fifteen, fifty-two, and eighty days at ice-box temperatures.

SUMMARY AND CONCLUSIONS

1. From the available information it is evident that infective dengue virus remains alive in its mosquito and animal hosts for much longer periods than in the dead tissues of these hosts, but that the duration of life in the latter under favorable conditions may be not inconsiderable.

2. The virus of dengue was apparently not preserved in an infective state in desiccated infective mosquitoes stored at 18° C. for four days or in frozen dried blood collected from experimental cases of dengue during the first day of fever when stored at 18° C. for four, fifty-two, and eighty days.

TABLE 7.—*Attempts to preserve dengue virus in frozen dried blood.*

Source of infective blood.			Period of storage in ice box.
Date.	Dengue patient.	Day of disease.	
			Days.
July 17, 1929	A.V. 21-M	First	4
July 17, 1929	do	do	4
June 26, 1929	A.V. 14-A	do	15
June 26, 1929	do	do	52
Sept. 10, 1929	A.V. 38-H	do	80

Subsequent test of dried blood for dengue virus.						
Date of inoculation into volunteers.	Volume of original inoculated.	Suspended in—	Filtered or unfiltered.	Susceptible volunteer.	Days observations after inoculation.	Result.
	cc.				*Days.*	
July 21, 1929	0.5	Salt solution	Filtered	A.V. 24-H	21	Negative.
July 21, 1929	0.5	do	Unfiltered	A.V. 26-T	13	Do.
July 11, 1929	1.5	do	Filtered	A.V. 28-P	24	Do.
Aug. 17, 1929	1.0	do	Unfiltered	A. V. 32-W	14	Do.
Nov. 29, 1929	1.0	do	do	A.V. 65-F	18	Do.

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FILTERABILITY OF DENGUE VIRUS OBTAINED FROM INFECTED MOSQUITOES

It was first demonstrated by Ashburn and Craig(1) in 1906 that infective virus from the blood of individuals suffering with dengue may be passed through diatomaceous filters. This observation has since been confirmed by other workers. For example, Koizumi, Yamaguchi, and Tonomura(2) in 1917 were successful in one of four tests; and Cleland, Bradley, and McDonald(3) in 1919 were able to pass dengue virus through filters in three of six experiments. Thus, it is apparent that while the virus, as it exists in the blood of dengue patients, is filterable, it is also to be noted that owing to several variable technical factors, filtration experiments with dengue virus, as with other viruses, are not always successful.

The present study was made to determine the filterability of the virus obtained from infected mosquitoes with the idea in mind that the results might give some indication as to whether the virus is similar during its development in both the insect and vertebrate hosts, or whether a different developmental cycle is required in the mosquitoes; also, if the virus obtained from the mosquitoes proved to be consistently filterable, it was planned to take advantage of this method for removing bacteria from suspensions of insects of various species fed on dengue blood, in order to test them for the presence of virus by inoculation into volunteers.

EXPERIMENTAL

METHODS AND MATERIALS

(*a*) *Source of dengue virus.*—The dengue virus fed to the various mosquito lots used in these experiments was a virulent strain (K) obtained originally in December, 1928, from a patient, Keller, who had dengue. This strain had been continued for a long period of time by repeated passages through man and mosquitoes.

(*b*) *Infection of mosquitoes.*—The mosquitoes of each lot were first given full feedings of infective blood from experimental cases of dengue during the first or second day of the disease.

In every instance the blood was proved infective at the time of feeding, by later transmission of the disease to susceptible volunteers either by mosquitoes of the same lots or, in the case of *Culex quinquefasciatus*, by *Aëdes* which had ingested the blood at the same time. *Aëdes ægypti* was used in all but two experiments, while *A. albopictus* was selected for one and *C. quinquefasciatus* for another.

(*c*) *Preparation of mosquito suspensions.*—In preparing the material for filtration, the mosquitoes were killed, and after having been ground up in a sterile mortar with sterile sand or powdered pyrex glass, they were suspended either in physiologic salt solution or in hormone broth.

(*d*) *Filtration.*—The suspensions of mosquitoes were immediately passed through cotton or were centrifugalized, to remove skeletal structures; and were then filtered through either Mandler or Berkefeld candles at a negative pressure of from 300 to 600 millimeters. The filters, which were examined for defects and tested to determine the pressure necessary to produce bubbles immediately after immersion in water, were found to differ considerably in resistance. Filtration of the mosquito suspensions required from ten to forty-five minutes. In every instance cultures showed the filtrates to be free of *Bacillus pyocyaneus* and the other culturable bacteria normally present in the mosquitoes.

(*e*) *Test for dengue virus in the filtrates.*—The various mosquito filtrates were tested for the presence of dengue virus by subcutaneous inoculation into susceptible volunteers, within two hours of the beginning of the experiments.

Experiment 1.—April 20, 1929. Fifty *Aëdes ægypti* of lot 10 were selected for this test. Mosquitoes of this lot had ingested infective blood fifty-two days previously from an experimental case (A.V. 4-Y) during the first day of dengue, and had transmitted dengue to volunteer A.V. 5-M. The mosquitoes were killed, placed in a sterile porcelain mortar, and macerated with 8 cubic centimeters of physiologic salt solution. This suspension was immediately passed through a "preliminary" type of Mandler filter (our No. 1) at a negative pressure of about 600 millimeters. When tested immediately afterwards it prevented the passage of a young culture of *Micrococcus melitensis*. The entire 6 cubic centimeters of filtrate obtained was then injected subcutaneously into a susceptible volunteer (A.V. 10-H), who after an incubation period of eight days, developed typical dengue fever, as is indicated in fig. 17. The diagnosis in this case was confirmed by subsequent transmission of the virus through *A. ægypti* of lot 17 to another volunteer (A.V. 12-S). Comment: In this experiment dengue virus from a saline suspension of *A. ægypti*, lot 10, passed through a "preliminary" type of Mandler filter.

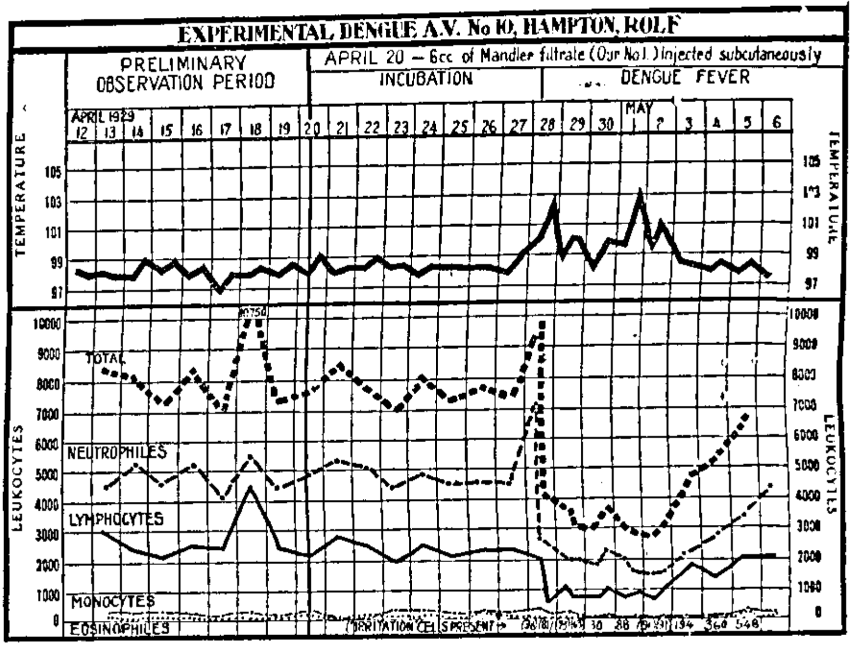

FIG. 17. Experimental dengue in American volunteer 10.

The positive results obtained in the first experiment suggested the possibility that suspensions of dengue virus from infected mosquitoes might be consistently filtered. Consequently, experiments were planned in an attempt to determine (*a*) whether or not the virus in *A. ægypti* is filterable and infective prior to the time when the mosquito is capable of transmitting the disease, and (*b*) whether or not the virus of dengue might not be developed and be demonstrable in the tissues of *Culex quinquefasciatus* even though mosquitoes of this species should be unable to transmit the infection.

Filtration experiments 2, 3, 4, and 5 were conducted with groups of mosquitoes from *A. ægypti* lot 25, all of which had taken full feedings of infective blood from an experimental case (A.V. 14–A) during the first twelve hours of dengue. At intervals of three, five, and fifteen days after the ingestion of this blood, groups of the mosquitoes were killed and suspended in salt solution. The filtrates obtained were inoculated subcutaneously into susceptible volunteers, while control mosquitoes from the same groups were allowed to feed on other volunteers. The infectivity of lot 25 was proved by the fact that after allowing the usual period for development of the virus, these mosquitoes fed on two men (A.V. 21–M and A.V. 22–M) and produced dengue in both instances.

Experiment 2.—June 29, 1929. Forty dengue-infected *A. ægypti* of lot 25, which had *fed on infected dengue blood only three days previously,* were ground in a mortar with sterile sand and suspended in 8 cubic centimeters of physiologic salt solution. After filtration through a wisp of cotton to remove the gross particles, this material was passed at a negative pressure of about 400 millimeters through a "preliminary" type of Mandler filter (our No. 10) which allowed air to bubble at about 4.4 pounds. Because of an accidental exposure of the material to possible bacterial contamination, it was filtered a second time. Five cubic centimeters of filtrate was obtained and 3 cubic centimeters of this was immediately injected subcutaneously into a susceptible volunteer (A.V. 20–B). Dengue did not result from this inoculation. Comment: As was to be expected, the control "biting" experiment with thirteen other "3-day" mosquitoes of this lot was negative, although dengue virus was known to have been present. Therefore, it seemed probable that failure of this filtration experiment might have been due to the fact that three days after feeding on infected blood the virus in the mosquitoes was not in a filterable stage, or that it was present in such small amounts as to be absorbed and retained in the filter, or that the technical procedure used and the double filtration might have prevented the passage of dengue virus regardless of its amount and source.

Experiment 3.—July 1, 1929. Forty dengue-infected *A. ægypti* of lot 25, which *had ingested dengue blood five days previously,* were ground up in a mortar with sterile sand and suspended in 6 cubic centimeters of physiologic salt solution. Filtration was carried out as in experiment 2, at a negative pressure of about 600 millimeters, through a "preliminary" type of Mandler filter (our No. 10), but the material was filtered only once. Three cubic centimeters of the filtrate was injected subcutaneously into a volunteer (A.V. 23–B), but failed to produce dengue. Feeding experiments with sixty-one additional "5-day" mosquitoes of the same lot also gave negative results. Comment: In this experiment the saline suspension was passed through the filter only once, rendering it improbable that double filtration was the only factor responsible for the negative result obtained in experiment 2.

Experiment 4.—July 11, 1929. One hundred fifty infective *A. ægypti* of lot 25 were used. These mosquitoes, which *had ingested infective blood fifteen days previously,* were proved to be infective, as they transmitted dengue by feeding to two susceptible volunteers (A.V. 21–M and A.V. 22–M). The one hundred fifty mosquitoes were ground in a mortar with sterile sand and suspended in 10 cubic centimeters of physiologic salt solution. This was divided into three portions of 4, 4, and 2 cubic centimeters, respectively, and one of the 4 cubic centimeter portions was used in this experiment. After adding 2 cubic centimeters of physiologic salt solution, the suspension, which represented sixty infected mosquitoes, was passed at a negative pressure of about 600 millimeters through a "preliminary" type of Mandler filter (our No. 10) which allowed air to bubble through at 4.4 pounds positive pressure. One cubic centimeter amounts of this filtrate were injected subcutaneously into each of two susceptible volunteers (A.V. 20–B and A.V. 23–B), but in both instances dengue failed to develop. Comment: In this experiment a saline suspension of virulent dengue virus from infective *A. ægypti* failed to pass through a "preliminary" type of Mandler filter. Therefore, it appeared probable that the negative results in experiments 2 and 3 were due to differences in technic or in the porosity of the filter candles, rather than to any variation in the filterability of the virus at different stages in the mosquito.

Experiment 5.—July 11, 1929. The 2 cubic centimeter portion of the suspension of *A. ægypti*, lot 25, mentioned under experiment 4, was diluted with 1 cubic centimeter of salt solution and passed at a negative pressure of about 600 millimeters through a coarse Berkefeld filter (our No. 3), which when tested later allowed air to bubble through at 6.8 pounds positive pressure. One cubic centimeter amounts of the bacteria-free filtrate were immediately injected subcutaneously into each of two susceptible volunteers (A.V. 24–H and A.V. 25–D), but in both instances they failed to produce dengue. Comment: In this experiment a saline suspension of virulent dengue virus from infective *A. ægypti* failed to pass through a coarse Berkefeld filter.

Experiment 6.—July 20, 1929. Twenty-four *A. ægypti* of lot 25, which had *ingested dengue blood twenty-four days previously*, and had been proved infective by feeding experiments, were combined with six other *A. ægypti* from infected lots 19 and 22 and suspended in 5 cubic centimeters of physiologic salt solution. Half of this mosquito suspension was passed at a negative pressure of 600 millimeters, through a "preliminary" type of Mandler filter (our No. 7), which when soaked in distilled water and tested immediately after use allowed air to bubble through at about 3.8 pounds positive pressure. Two and three-tenths cubic centimeters of the bacteria-free filtrate were injected subcutaneously into a susceptible volunteer (A.V. 20–B); and failed to cause dengue. Comment: The virus in a saline suspension of mosquitoes failed to pass through a Mandler filter (our No. 7).

Experiment 7.—July 20, 1929. The remaining 2.5 cubic centimeter portion of saline suspension of dengue-infected *A. ægypti*, mentioned under experiment 6, was passed at a negative pressure of 600 millimeters through a "preliminary" type of Mandler filter (our No. 8). This filter, when tested after immersion in distilled water July 13, allowed air to bubble through at a positive pressure of 6.4 pounds. The bacteria-free filtrate was injected subcutaneously into a susceptible volunteer (A.V. 23–B) and did not cause dengue. Comment: The infective virus in this saline suspension of mosquitoes failed to pass through a Mandler filter (our No. 8).

Experiment 8.—July 29, 1929. Thirteen dengue-infected *A. ægypti* of lot 25, which *had ingested dengue blood thirty-three days previously*, were combined with nineteen infected *A. ægypti* of lot 30 and eighteen of lot 32 and were suspended in about 10 cubic centimeters of salt solution. The material from the suspension of these fifty mosquitoes was passed at 400 millimeters of vacuum through a preliminary Mandler filter (our No. 7). About 6 cubic centimeters of bacteria-free filtrate was obtained and 4 cubic centimeters of this was injected subcutaneously into a susceptible volunteer (A.V. 27–P), but dengue did not result. Comment: In this experiment infective virus failed to pass through a preliminary Mandler filter.

Two other lots of mosquitoes, *A. albopictus* lot 29, and *A. ægypti* lot 30, were used later in filtration experiments 9 and 10. These mosquitoes had ingested blood from an experimental case (A.V. 22–M) July 16, during the latter half of the first day of the disease. Both of these lots of mosquitoes transmitted dengue later when numbers of them were fed on susceptible volunteers.

Experiment 9.—July 21, 1929. Fifteen *A. albopictus* of lot 29, which had *fed on infective dengue blood five days previously,* were macerated with sterile sand and 1.5 cubic centimeters of salt solution. The suspension was filtered at a negative pressure of about 600 millimeters through a regular Mandler filter (our No. 9), which when tested July 12 had allowed air to bubble through at a positive pressure of 6.4 pounds. Half of the bacteria-free filtrate (0.75 cubic centimeter) was immediately injected subcutaneously into a susceptible volunteer (A.V. 27–P) with negative results. Comment: This Mandler filtrate did not contain virulent dengue virus.

Experiment 10.—July 21, 1929. Seven *A. ægypti* of lot 30, which had *fed on infective dengue blood five days previously,* were macerated with sand and 3 cubic centimeters of salt solution. This suspension was filtered at a negative pressure of about 600 millimeters through a "preliminary" grade of Mandler filter (our No. 7). One and one-half cubic centimeters of the bacteria-free filtrate were injected subcutaneously into a susceptible volunteer (A.V. 25–D), with negative results. Comment: This Mandler filtrate did not contain infective dengue virus.

In experiments 11 and 12 the mosquitoes used were *Culex quinquefasciatus* of lot 31, and *Aëdes ægypti* of lot 32. Both had ingested dengue-infected blood from the same experimental case (A.V. 21–M) during the first day of the disease. Lot 32 *A. ægypti* were later proved to be infected, as they were able to transmit the disease to a susceptible volunteer by feeding.

Experiment 11.—July 20, 1929. Twenty-five *C. quinquefasciatus* of lot 31, which had *ingested infective blood four days previously,* were killed, macerated with sterile sand, and suspended in 4 cubic centimeters of physiologic salt solution. The mosquito suspension was filtered at about 600 millimeters negative pressure through a "regular" type of Mandler filter (our No. 12), which when tested on the same day allowed air to bubble through at 6.6 pounds positive pressure. Three cubic centimeters of the bacteria-free filtrate was injected subcutaneously into a susceptible volunteer (A.V. 28–P) with negative results. Comment: The fact that this filtrate was free from infective dengue virus does not prove that the *C. quinquefasciatus* of lot 31 did not harbor the virus, as filtrates from *A. ægypti* known to be infected were also free from virus in several instances.

Experiment 12.—August 17, 1929. One hundred infective *A. ægypti* of lot 32, which *thirty-one days previously had fed on dengue blood* similar to that ingested by *C. quinquefasciatus* lot 31, were killed, freed of wings and legs, and ground in a sterile glazed porcelain evaporating dish with 4 cubic centimeters of hormone broth. The suspension was centrifugalized at low speed for five minutes to remove gross particles and was divided into two portions of 2 cubic centimeters each. One of these portions was passed at about 300 millimeters negative pressure, through a "preliminary" type of Mandler filter (our No. 7), after the filter had been saturated by drawing hormone broth through it. About 5 cubic centimeters of filtrate was obtained, and half of this (2.5 cubic centimeters) was inoculated subcutaneously into a susceptible volunteer (A.V. 30–L), but the injection failed to produce dengue. Comment: The use of hormone broth in this experiment did not make it possible to pass the virus through the filter.

Experiment 13.—August 17, 1929. The other 2 cubic centimeter portion of the mosquito suspension in hormone broth, described under experiment 12, was passed, at about 300 millimeters negative pressure, through a Berkefeld "V" filter (our No. 19), which after immersion in water allowed air to bubble at about 9.3 pounds positive pressure. The 2 cubic centimeters of mosquito suspension was added, a drop at a time, to the top of the candle, and as none of it came through the filter in fifteen minutes, an additional 3 cubic centimeters of hormone broth was added in an attempt to wash the virus through. As a result 2 cubic centimeters of bacteria-free filtrate was obtained, of which 1.5 cubic centimeters was immediately inoculated subcutaneously into a susceptible volunteer (A.V. 29–J). After an incubation period of nine and one-fourth days this volunteer developed symptoms of typical dengue fever. The specific nature of the infection was further proved by subsequent passage of the virus through *A. ægypti* lot 49 to another volunteer (A.V. 33–B). Conclusions: In this experiment dengue virus from the hormone-broth suspension of *A. ægypti* lot 32 passed through a "V" (coarse) type of Berkefeld filter.

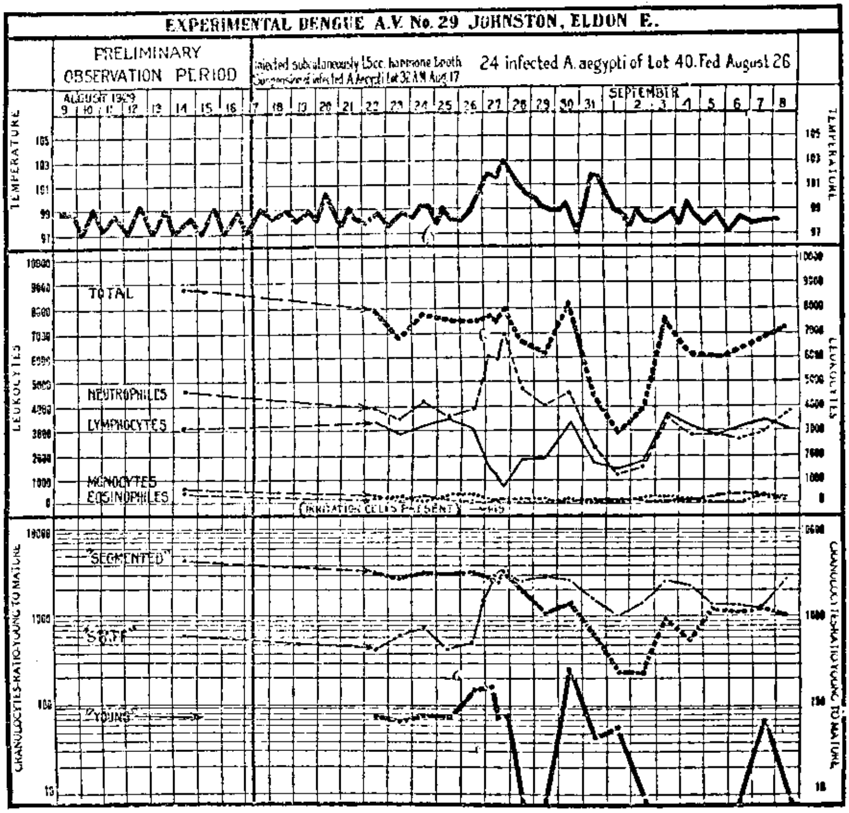

FIG. 18. Experimental dengue in American volunteer 29.

Experiment 14.—November 29, 1929. Fifty dengue-infected *A. ægypti* of lot 95, which thirty-three days previously had ingested dengue blood from an experimental case (A.V. 48–H), were killed, ground up, and suspended in 4 cubic centimeters of hormone broth. The material was passed at 600 millimeters negative pressure through a "regular" type of Mandler filter (our No. 23) in ten minutes. One cubic centimeter of the bacteria-free filtrate was injected subcutaneously into a susceptible volunteer (A.V. 64–C). Dengue did not result from this inoculation.

Experiment 15.—November 30, 1929. Fifty infected *A. ægypti* of lot 104, which *twenty-nine days previously* had fed on blood from an experimental case (A.V. 55–S) during the first day of dengue and had later transmitted dengue by biting, were killed, ground up with powdered pyrex glass, and suspended in 2 cubic centimeters of hormone broth. The suspension was centrifugalized slowly for one minute and the supernatant fluid was passed at 600 millimeters negative pressure through a Berkefeld W filter (our No. 20) which allowed air to bubble at 12.4 pounds pressure. After about thirty minutes an additional 1 cubic centimeter of hormone broth was passed through the filter. The entire time of filtration was forty-five minutes and the total amount of filtrate 1.5 cubic centimeters. Injection of 1 cubic centimeter of this bacteria-free filtrate failed to produce dengue in a volunteer (A.V. 63–B), but as this volunteer was later found to be immune the experiment has no significance.

Experiment 16.—March 14, 1930. Thirty-one dengue-infected *A. ægypti* of lot 138 and sixty-nine of lot 167 were killed, ground up with powdered pyrex glass, and suspended in 5 cubic centimeters of physiologic salt solution. The suspension was placed in the mantle of a Berkefeld V filter (our No. 19) which allowed air to bubble through at 9.3 pounds pressure, and suction (600 millimeters) was applied for five minutes. Then 3 cubic centimeters of salt solution was added and about 1.5 cubic centimeters of filtrate was obtained in five minutes. One cubic centimeter of the bacteria-free filtrate was injected subcutaneously into a susceptible volunteer (A.V. 87–R). After nine and one-quarter days incubation, dengue developed from this inoculation, as shown in fig. 19. Comment: Dengue virus from this saline suspension of *A. ægypti* passed through a Berkefeld V filter.

DISCUSSION

It has been shown by these experiments that the virus of dengue as it exists in infective mosquitoes may, under certain circumstances, be passed through diatomaceous earth filters. However, as has been observed in filtration experiments with the blood of dengue patients and with materials containing other "filterable" viruses, such positive results were not obtained in all tests.

Because of the many variable factors which might have influenced the results, it was not possible to determine with exactness just what combination of conditions are necessary to ensure

TABLE 8.—*Filterability of dengue virus from mosquitoes (Berkefeld and Mandler filters).*

Experiment No.	Mosquitoes used.		Preparation of suspension.			Filtration of suspension.			Filtrate.[a]		
	Species.	Days since ingestion of virus.	Number of mosquitoes used.	Amount of physiologic salt solution.	Amount of harmone broth.	Filter used.	Negative pressure used.	Time required.	Amount obtained.	Amount injected into volunteer.	Results of injection.
				cc.	*cc.*		*mm.*	*Min.*	*cc.*	*cc.*	
1	*Aëdes ægypti*	52	50	8		Mandler No. 1 (preliminary)	600	30	6	6	Dengue fever in 8 days.
2	do	3	40	8		Mandler No. 10 (preliminary)	400	20	5	3	Negative.
3	do	5	40	6		do	600	20	4	3	Do.
4	do	15	60	6		do	400	25	4	1 1	Do. Do.
5	do	15	30	3		Berkefeld No. 3 "V"	600	25	2.5	1	Do.
6	do	24 67 63	12 1 2	2.5		Mandler No. 7 (preliminary)	600	15	2.3	1 2.3	Do. Do.
7	do	24 67 63	12 1 2	2.5		Mandler No. 8 (regular)	600	20	1.8	1.5	Do.
8	do	33 13 12	13 19 18	10		Mandler No. 7 (preliminary)	400	20	6	4	Do.
9	*Aëdes albopictus*	5	15	1.5		Mandler No. 8 (regular)	600	20	1	0.75	Do.
10	*Aëdes ægypti*	5	7	3		Mandler No. 7 (preliminary)	600	15	1.8	1.5	Do.
11	*Culex quinquefasciatus*	4	25	4		Mandler No. 12 (regular)	600	20	3.3	3	Do.
12	*Aëdes ægypti*	31	50		2	Mandler No. 7 (preliminary)	300	15	[b] 5	2.5	Do.
13	do	31	50		[c] 2	Berkefeld No. 19 "V"	300	25	2	1.5	Dengue fever in 9½ days.
14	do	33	50		4	Mandler No. 23 (regular)	600	10	2	1	Negative.
15	do	29	50		[d] 2	Berkefeld No. 20 "W"	600	45	1.5	1	Negative (immune).
16	do	29 16	69 31	8		Berkefeld No. 19 "V"	600	10	1.5	1	Dengue fever in 9½ days.

[a] No demonstrable growth was obtained in culture of the filtrates.

[b] In experiment 12 the filter was first saturated with hormone broth, and drained as completely as possible before use; it is apparent that the filter still contained broth in addition to that used in the mosquito suspension.

[c] Three added later.

[d] One added later.

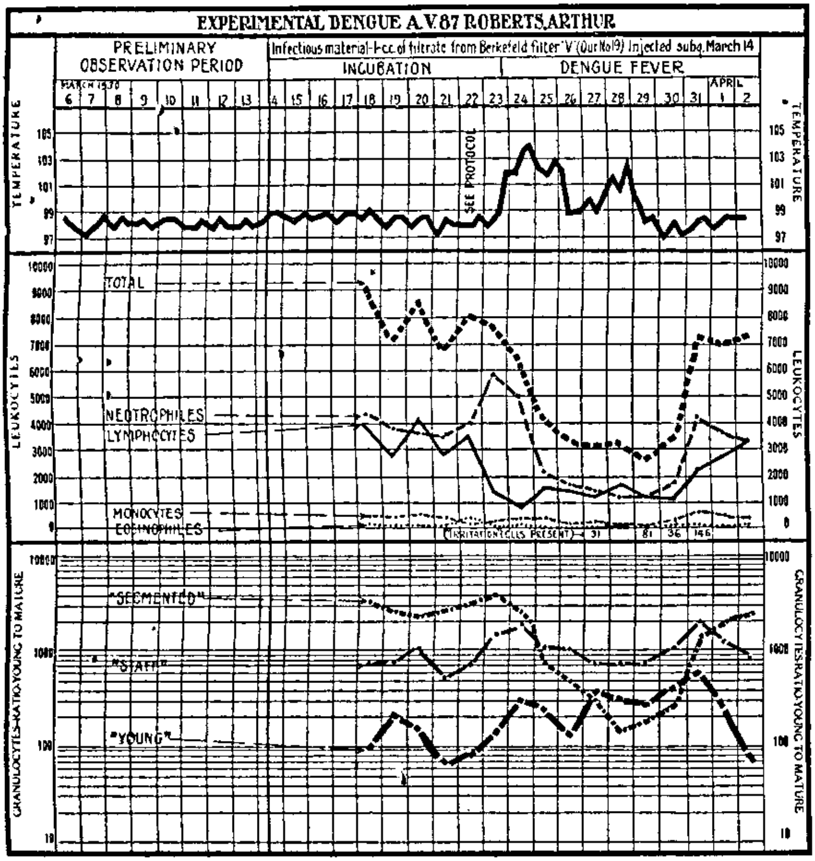

FIG. 19. Experimental dengue in American volunteer 87.

filtration of the virus at all times. It will be noted in Table 8 that in the three successful filtration experiments the conditions were as follows: (*a*) In the first test fifty mosquitoes which had been infected for fifty-two days were suspended in 8 cubic centimeters of salt solution, passed at 600 millimeters through a Mandler filter, and 6 cubic centimeters of this filtrate was inoculated into the volunteer; (*b*) in the second, fifty mosquitoes were suspended in 2 cubic centimeters of hormone broth, passed at 300 millimeters through a Berkefeld "V" filter and washed through by adding 3 cubic centimeters more broth, and then 1.5 cubic centimeters of the 2 cubic centimeters of filtrate was injected into the volunteer; while (*c*) in the third test, one hundred mosquitoes suspended in 5 cubic centimeters of salt solution were passed through a Berkefeld "V" filter with an additional 3 cubic centimeters of salt solution and 1 cubic centimeter of the filtrate was used for the inoculation of the volunteer. Since the type of these filters, the negative pressure applied, the reactions, and the materials used in preparing the mosquito suspensions were duplicated in negative experiments, it appears possible that the most important factor was the relative amounts of virus present in the suspensions. It is believed that in the negative experiments the suspensions contained such small amounts of virus that they were adsorbed and held in the candles, and that the use of larger amounts of virus might have given positive results. In the three successful tests the concentration of virus was probably greater because of the larger amounts of mosquito suspensions used. In this connection it appears of some significance that in the three volunteers infected by these filtrates, the incubation periods were eight, nine and one-fourth, and nine and one-fourth days, respectively, or longer than the average. It seems reasonable to suspect that this prolongation of the period of incubation might have resulted because small amounts of virus were present in the filtrates injected.

Note.—It is of interest to add that since the first of these successful experiments was reported(4) an article has been published by Sawyer and Frobisher(5) announcing the fact that the virus of yellow fever in infected *Aëdes* mosquitoes is filterable.

SUMMARY AND CONCLUSIONS

1. Sixteen filtration experiments are reported, in which attempts were made to pass dengue virus from suspensions of infected mosquitoes through various types of diatomaceous earth filters.

2. The variable results obtained in these experiments agree in general with the observation of others concerning the filterability of dengue virus in blood.

3. In three experiments, one with a Mandler filter and two with Berkefeld "V" filters, infective virus was shown to be present in the filtrates.

4. The inoculation of these three filtrates into volunteers resulted in dengue fever after incubation periods of eight, nine and one-fourth, and nine and one-fourth days, respectively.

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EXPERIMENTAL DENGUE IN MAN

It is commonly believed that many of the fevers of the Tropics masquerade under the diagnostic term "dengue." This state of confusion is attributable mainly to the lack of any specific diagnostic test with which to differentiate dengue from similar or related fevers of undetermined origin. For this reason it is at least possible that studies based only on the clinical signs and symptoms observed in naturally acquired infections might fail to indicate the true clinical picture of dengue.

The following analysis of clinical symptoms observed in over eighty cases of experimental dengue is considered of interest and value mainly because of the fact that they were all produced by a single strain of dengue virus. While the possibility, of course, exists that more than one type of denguelike virus may exist in nature, the fact that 93 per cent of thirty-three Filipinos residing in endemic areas were shown to be immune to experimental infection, indicates that the virus used in this work is probably immunologically similar to or identical with the dengue virus prevalent in the Philippines.

The diagnosis of experimental infections produced by this virus was confirmed by the appearance of distinctive symptoms, typical leukocyte reactions, and in the majority of instances by subsequent transmission of the infection through mosquitoes to other individuals. It is of interest to note that even with this single strain of virus, wide variations occurred in the character and severity of the disease.

It was considered possible that these variations might have resulted either from differences in the amounts of virus introduced into the volunteers; from changes in the virulence of the virus due to the various experimental procedures or passages; or from individual differences in the susceptibility of the volunteers infected. It was apparent that the latter factor alone was of great importance. As infections produced in May, 1930. did not differ noticeably from others caused by the same strain of virus a year and a half earlier, it is obvious that the virulence was neither increased nor decreased appreciably. The fact that infections similar in type and severity were caused by the feeding of either small or large groups of infected mosquitoes (Table 9) indicates that within these limits, the dose of virus failed to influence the character of the symptoms. However, it was noted in certain filtration experiments, and in others where virus was transferred by the interrupted feeding of mosquitoes that the incubation periods were slightly prolonged. It was considered probable that this was due to the introduction of minute amounts of virus into the volunteers.

Therefore, it is believed that the clinical variations observed in the entire group of experimental cases were due mainly to differences in individual susceptibility of the volunteers to dengue.

THE PERIOD OF INCUBATION

It has been estimated that the period required for the incubation of dengue virus in man is from two to ten days, but Armstrong(2) states that it is usually from three to six days. Cleland(3) (1923) is of the opinion that "the incubation period of the natural disease may be set down as being usually from 3 to 8 days, with outside limits of 2½ and 15 days. Such an incubation period is consistent with clinical experience. Instances in which the time appears to have been less than 2 days can probably all be explained in some other way." He reported an incubation period of fifteen days in one volunteer who was inoculated with blood drawn from a case in the fourth day of the disease. Siler, Hall, and Hitchens,(1) (1926) from an analysis of their experimentally infected cases, arrived at the following conclusions:

> The average incubation period in our series was six and one-twentieth days; in 25 per cent it was less than five days; in 60 per cent, less than six days; in 75 per cent, less than seven days; and in 90 per cent, less than eight days. Incubation periods of more than ten days must be very rare in naturally acquired cases.

The results obtained in the present series, shown in Table 10, give an average incubation period of 5.66 days, with 83.75 per cent of eighty infections occurring between the fourth and seventh days after inoculation. The maximum period observed was between eleven and thirteen days; and the minimum was between three and four days. However, it is to be noted that only one case occurred in each of the latter groups, and that the infection with the prolonged incubation period was the result of interrupted feeding of *Culex quinquefasciatus*.

TABLE 9.—*Experimental dengue in man. A camparison of cases produced by small and large numbers of mosquitoes.*

Number of mosquitoes fed.	Lot No.	Volunteer No.	Incubation period.	Fever.		Leukopenia.		Rash.			Character of attack.		
				Duration.	Height.	Duration.	Lowest point.	None.	Primary.	Secondary.	Mild.	Average.	Severe.
			Days.	*Days.*		*Days.*							
7	9	I.V. 1-T	5.25	5.5	103.6	5	4,700	*					*
6		I.V. 2-B	5.25	4	101.2	2	4,700	*				*	
6		I.V. 3-P	5.25	6	102.4	6	4,600	*					*
5		I.V. 4-B	4?	4 ?	101	4	4,100	*				*	
9	30	A.V. 20-B	6	3.5	100.4	3.5	4,200		*		*		
8	32	A.V. 28-P	6	4.5	102.6	5	3,700	*				*	
10	52	A.V. 46-L	5.5	5.5	102.4	9	3,250			*		*	
9		A. V. 43-D	4.5	5.5	103.6	8	2,600		*?			*	
6		A.V. 41-F	4.5	6	103.2	5	2,850		*	*		*	
8		A.V. 44-F	5	5.5	101.6	4	3,300			*	*		
8	71	A.V. 55-S	5	5	104	10	2,950	*				*	
10	86	A.V. 50-C	5.5	4	104	5	2,800		*	*		*	
10	112	A.V. 65-F	6	5.5	102.4	7	3,150		*	*			*
7	137	A.V. 70-S	5	5	102.8	4	3,150		*	*		*	
6		A.V. 71-W	6	2	102.6	7	2,900			*	*		
7.7 (average)			5.25	4.8	102.4	5.7	3,530	* 40	* 40	* 47	* 20	* 60	* 20
56	2	A.V. 4-Y	7.5	3	104	2.5	3,700	*				*	
56	3	A.V. 3-A	6	5	103	5.5	3,350	*					*
85	18	A.V. 13-W	4.5	5	102	5	2,400	*				*	
139	10	A.V. 5-M	5	4.5	104	4	2,950			*			*
82	35	A.V. 31-D	4	5.5	104	9	2,500			*			*
64	71	A.V. 48-H	5	5.5	103.2	9	2,050			*		*	
109	114	A.V. 66-W	5	4	101.4	3	4,150		*	*		*	
84	116	A.V. 61-J	5	5	104	8	2,950			*			*

58	117	A.V. 64-C	6	4.5	102.2	6	2,600	--------	*	--------	--------	*	--------
89	137	A.V. 67-M	5	6	103	5	2,900	--------	*	*	--------	*	--------
61	137	A.V. 76-M	6	3.5	101.2	7	3,300	--------	--------	*	*	--------	--------
60	138	A.V. 75-P	5	5.5	103.8	7	2,150	--------	*	--------	--------	*	--------
110	139	A.V. 68-J	5.5	5.5	103.8	5	3,400	--------	*	*	--------	*	--------
87	162	A.V. 77-E	4.5	6.5	102	7	1,750	--------	*	*	--------	*	--------
108	167	A.V. 82-F	4.5	6.5	101.4	3	4,100	--------	--------	*	--------	*	--------
83.2 (average)	--------	--------	5.2	5.0	102.9	5.7	2,937	*20	*40	*66	*6.6	*6	*26.6

* Per cent.

TABLE 10.—*The incubation periods in experimental dengue fever observed in eighty cases.*

Days of incubation.	Cases.	Per cent.
3–4	1	1.25
4–5	15	18.75
5–6	36	45.00
6–7	16	20.00
7–8	5	6.25
8–9	2	2.50
9–10	4	5.00
11–13	1	1.25
Total	[a]80	100.00

[a] Average, 5.66 days incubation.

PRODROMAL SYMPTOMS AND ONSET

In 58 per cent of the sixty cases included in fig. 20, the onset of the disease was abrupt, beginning with fever or severe headache, or more rarely, with a distinct chill. In a few of the remaining 42 per cent of cases with gradual onset, mild prodromal symptoms such as malaise, weakness, chilliness, and indefinite pains and aches were noticed for a day or so before the definite attack. The symptoms at onset consisted of fever, headache, flushing of the face, aching of the eyes, pains in the back and extremities, and malaise. Chilly sensations or even chills were not uncommon. In certain cases an initial rash was observed. Leukopenia was not present until the first or second day following the onset, although an increase in the immature with a corresponding decrease in the mature granulocytes was not uncommonly observed on the day preceding fever.

THE FEVER

The results obtained experimentally by Siler, Hall, and Hitchens,(1) and during the present studies, show that the temperature reactions observed in dengue may differ so greatly in type, height, and duration that this symptom considered by itself has no specific, diagnostic significance. Among the experimental infections considered in this report patients were seen who had "two-day fever," "three-day fever," and so on, including a few cases with "seven-day fever." As shown in Table 11, the duration of the fever in eighty-one experimental cases was

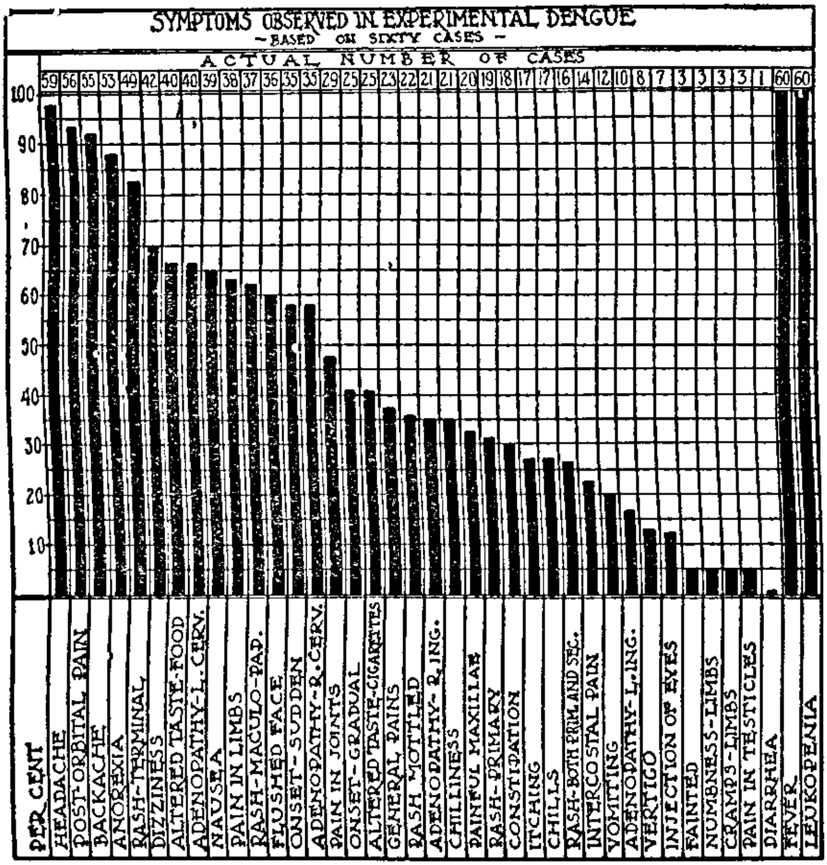

FIG. 20. Symptoms observed in experimental dengue.

from one to nine days, although in the average case the interval between the beginning and end of the fever was 4.8 days. There was also considerable variation in the maximum temperatures recorded. In 52 per cent the temperature was 103° F. or above, while the highest temperature reached in 48 per cent was 102.8° F. The so-called "saddle back" or intermittent type of fever, which has been considered as characteristic of dengue, was observed in certain cases but was by no means constant. In these cases the interval of reduced fever coincided with a period of relief from the subjective symptoms, while the return of the fever was usually accompanied by the symptoms noted in the first stage of the disease.

There was no indication that the fever varied in duration in any fixed relation to the incubation period, as has been observed in certain other diseases. As shown in Table 13, cases with both long and short incubation periods had fevers of similar duration.

The subjective symptoms of dengue lasted from one to nine days, during which the various symptoms noted at onset often became more pronounced. Fever, leukopenia, headache, postorbital pain, generalized aches and pains particularly of the back, anorexia, nausea, dizziness, abnormal sense of taste, adenopathy, and terminal rash were the commonest symptoms observed. Certain of these symptoms are of sufficient importance to be considered separately.

TABLE 11.—*Duration of fever in eighty-one cases of experimental dengue fever.*

Days of fever.[a]	Cases.	Per cent.
1-2	2	2.47
2-3	3	3.71
3-4	10	12.35
4-5	16	19.75
5-6	37	45.68
6-7	11	13.58
7-8	1	1.23
8-9	1	1.23
Total	81	100.00

[a] Average, 4.8 days fever.

TABLE 12.—*Maximum temperature observed in eighty-one cases of experimental dengue fever.*

Maximum temperature.[a]	Cases.	Per cent.
°F.		
100-101	2	2.47
101-102	12	14.82
102-103	25	30.86
103-104	28	34.57
104-104.6	14	17.28
Total	81	100.00

[a] Average temperature 102.82° F.

TABLE 13.—*Comparison of the incubation periods and the duration of fever in eighty cases of experimental dengue.*

Incubation period.	Days of fever.							
	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9
Days.								
3-4				1				
4-5	1			1	7	5		1
5-6			4	8	20	4		
6-7		1	2	4	7	1	1	
7-8		1	2	2				
8-9					2			
9-10	1	1			1	1		
10-11								
11-13			1					
Total	2	3	9	16	37	11	1	1

PROGRESS OF THE DISEASE

Headache and generalized pains.—Beside the fever and leukopenia, which were noted in all cases, the commonest symptoms were headache, postorbital pains, and backache, which occurred in over 90 per cent of the sixty individuals considered. More than 60 per cent complained of pains in the limbs and 45 per cent had painful joints. However, while these symptoms were far from mild, none of them were severe enough to warrant application of the old name, "break-bone fever."

Skin rashes.—There seems to be a general belief among clinicians that the rash is one of the most characteristic symptoms of dengue; and consequently the clinical diagnosis frequently depends on the presence or absence of this sign. Siler, Hall, and Hitchens (1) noted that in only two, or 4 per cent, of their experimental cases was there failure to observe an eruption at some time during the course of the disease. Six of their cases had an initial eruption, but no terminal rash, and in seven instances the conditions were reversed. Twenty-three showed both initial and terminal rashes. It was stated that "evidently the eruption is a very variable symptom, requiring careful and frequent observation if all of its details are to be noted; in some cases it may fail altogether to develop."

In the present study, the initial rash, usually an erythematous flush or mottling, was observed in only 38.8 per cent of the cases (Table 14), and it was frequently difficult to differentiate this rash from other skin eruptions, particularly the heat rashes. Some of the primary rashes lasted for only a few hours, and for this reason it is considered possible that a few were not observed. Because of the difficulty experienced in recognizing the initial rash, and because of its evanescent character, this sign was not considered of definite diagnostic value. The terminal rash, which was noted in 69.5 per cent of the cases, was of more importance. It was usually of the maculo-papular variety, and in a few cases it was followed by desquamation and in some instances by itching of the palms of the hands and soles of the feet. Although this rash might be confused with other skin eruptions, its occurrence in a febrile condition, accompanied by characteristic symptoms of dengue, including leukopenia, is of considerable diagnostic significance.

Glandular enlargement.—As indicated in fig. 20, a large proportion of the cases had enlargement of the cervical or inguinal lymph glands. The cervical glands were most frequently affected, which indicates that the location of the infective mosquito bite was probably not a determining factor, since most of the volunteers were bitten on the leg. In over 30 per cent of the cases there was some tenderness in the region of the parotid glands and the patients complained that their "jaws ached." However, no enlargement of the gland was noted and the pains may have originated in the joint. Two cases were seen in Ifugao volunteers of definite parotid involvement, one of whom had such marked enlargement of the glands as to suggest temporarily that he might have mumps. Five per cent of the cases complained of swelling or tenderness of the testicles.

Gastrointestinal symptoms.—A large proportion of the patients complained of loss of appetite, and at least 65 per cent had perversions of taste for various foods or for cigarette smoke. Nausea was also common, particularly early in the disease, and vomiting occurred in 20 per cent of the cases. Constipation was noted in 30 per cent of the infections.

Genitourinary system.—In a few cases a trace of albumin was noted temporarily during the fever.

Circulatory system.—The pulse rate during the disease was usually low in proportion to the fever.

Hæmorrhage.—Among the Americans only one case of dengue had epistaxis; and none had hæmorrhage from other parts of the body. On two occasions severe nose bleed was observed in an Ifugao volunteer during the height of dengue fever.

TABLE 14.—*Experimental dengue in man.*[a]

Infection of volunteers.							Character of infection.						
	Infected mosquitoes used.						Fever.		Leukopenia.		Rash.		
Volunteer No.	Species.	Lot No.	Age of virus.	Number fed or injected.	Date fed or injected.	Incubation period in volunteers.	Duration.	Maximum temperature.	Duration.	Minimum count.	Primary.	Secondary.	Character of attack.
			Days.			*Days.*	*Days.*		*Days.*				
1	*Aëdes ægypti*	1	16	17	Dec. 31, 1928	9	2	102.4	3	3,150	×	×	Mild.
2	do	1	15	15	Dec. 31, 1928	7	3.25	102.6	4.5	3,250	×	×	Average.
3	do	3	40	56	Feb. 19, 1929	6	5	103	5.5	3,350	0	0	Severe.
4	do	2	50	56	Feb. 19, 1929	7.5	3	104	2.5	3,700	0	0	Average.
5	do	10	40	139	Apr. 8, 1929	5	4.5	104	4	2,950	0	×	Severe.
6	do	9	45	[illegible]9	Apr. 11, 1929	5	4.5	101	6	2,900	×	0	Average.
7	do												
8	*Aëdes ægypti*	9+10	59+57	29	Apr. 25, 1929	5	5.25	103.2	6.5	2,850	0	0	Do.
9	do	12	22	15	May 8, 1929	6	4.5	102.8	7	2,850	0	×	Do.
10	do	10	52	50	Apr. 20, 1929	8	5.5	103.2	6	2,800	×	0	Severe.
11											(b)		
12	*Aëdes ægypti*	17	12	17	May 11, 1929	6	5	102	6	2,950	0	0	Average.
13	do	18	16	85	May 17, 1929	4.5	5	102	5	2,400	0	0	Do.
14	do	19	37	12	May 20, 1929	5.5	5	103.8	8	2,850	0	×	Severe.
15													
16													
17													
18													
19													

[a] Average incubation period, 5.66 days; average duration of leukopenia, 6.2 days; average duration of fever, 4.8 days; primary rash, 38.8 per cent; secondary rash, 69.5 per cent; mild attacks, 13.6 per cent; average attacks, 70.4 per cent; severe attacks, 16.0 per cent.

[b] Slight.

TABLE 14.—*Experimental dengue in man*—Continued.

Infection of volunteers.						Incubation period in volunteers.	Character of infection.						Character of attack.
Volunteer No.	Infected mosquitoes used.						Fever.		Leukopenia.		Rash.		
	Species.	Lot No.	Age of virus.	Number fed or injected.	Date fed or injected.		Duration.	Maximum temperature.	Duration.	Minimum count.	Primary.	Secondary.	
			Days.			*Days.*	*Days.*		*Days.*				
20	*Aëdes ægypti*	30	13	9	July 29, 1929	6	3.5	100.4	3.5	4,700	×	×	Mild.
21	do	25	15	15	July 11, 1929	5	5.5	104	6	2,750	0	0	Averge.
22	do	25	15	14	July 11, 1929	4.5	5	103	7	2,150	0	0	Do.
23	*Aëdes albopictus*	29	13	12	July 29, 1929	7	4.5	101.4	6	2,150	×	×	Do.
24	do	29	22	10	Aug. 7, 1929	5.5	3.5	102	4	5,100	0	×	Mild.
25	do	29	21	3	Aug. 6, 1929	6.25	5.5	103	7	3,200	0	×	Average.
26	do	29	13	8	July 29, 1929	4.5	1.5	101	4.5	3,900	×	0	Mild.
27	do	29	21	1	Aug. 6, 1929	5	6	103.2	6	2,100	0	×	Average.
28	*Aëdes ægypti*	32	12	8	July 29, 1929	6	4.5	102.6	5	3,700	0	0	Do.
29	do	32	30	50	Aug. 17, 1929	9	5.5	103	7	2,950	0	0	Do.
30	do	41	20	16	Aug. 26, 1929	4.25	4	103	7	3,450	0	×	Do.
31	do	35	22	82	Aug. 26, 1929	4⅓	5.5	104	9	2,500	0	×	Severe.
32	*Aëdes albopictus*	43	14	22	Aug. 26, 1929	5	5	104.6	7	2,150	0	0	Do.
33	*Aëdes ægypti*	49	19	33	Sept. 14, 1929	4.5	6	102	6	2,500	0	0	Average.
34	*Aëdes albopictus*	43	19	2	Aug. 31, 1929	5	5	103.6	10	2,400	×	×	Severe.
35	*Aëdes ægypti*	92–*a*, *b*, *c*, *d*, *e*, *f*, *g*.	17–21	7	Aug. 26, 1929	6.5	5	102	8	3,600	×	×	Average.
36	do	44, 45, 46, 47	29–31	28	Sept. 21, 1929	5	5	103.4	7	2,750	×	×	Severe.
37	do	53	15	42	Sept. 14, 1929	7	4	103.4	7	2,700	0	×	Average.
38	do	92–*a*, *b*, *c*, *d*, *e*, *f*, *g*.	31–37	17a, 24b, 37c, 36d, 29e, 23f, 37g.	Sept. 5, 1929	5	5.5	104	9	3,100	×	×	Do.

39	do	44, 45, 46, 47	14–16	47	Sept. 5, 1929	6	6	103.4	8	2,900	×	0	Severe.
40													
41	*Aëdes ægypti*	52	37	6	Oct. 7, 1929	4.25	6	103.2	5	2,850	×	×	Average.
42	do	92[c]	41–44	28	Sept. 21, 1929	5.5	6	104	11	2,650	0	×	Severe.
43	do	52	82	9	Oct. 2, 1929	4.25	5.5	103.6	8	2,600	0	×	Average.
44	do	52	41	8	Oct. 11, 1929	5	5.5	101.6	4	3,300	0	×	Mild.
45	*Aëdes albopictus*	58	26	3	Oct. 2, 1929	5	4	102	8	3,600	0	×	Average.
46	*Aëdes ægypti*	52	32	10	Oct. 2, 1929	5.5	5.5	102.4	9	3,250	0	0	Do.
47	do	69	14	50	Oct. 7, 1929	5	3.5	101	9	4,550	0	×	Mild.
48	do	71	25	64	Oct. 21, 1929	5	5.5	103.2	9	2,050	0	×	Average.
49													
50	do	86	20	10	Nov. 7, 1929	5.5	4	104	5	2,800	×	×	Do.
51	do	86	14	14	Oct. 27, 1929	5.5	6	104.4	9	2,050	0	×	Do.
52													
53	*Aëdes ægypti*	82, 83, 84, 85, 88, 89.	13–18	5, 15, 4, 6, 15, 9.	Oct. 28, 1929	6½	5	103.6	8	3,200	0	0	Do.
54	*Culex quinquefaciatus*	97	0	54	Oct. 31, 1929	13	3.5	103.4	6	3,100	0	×	Do.
55	*Aëdes ægypti*	71	31	8	Oct. 26, 1929	5	5	104	10	2,950	0	0	Do.
56	do	104	15	25	Nov. 16, 1929	7	2.5	100.2	5	4,350	×	×	Mild.
57	do	96	12	30	Nov. 11, 1929	5	[illegible].5	101.6	8	2,300	×	×	Severe.
58	do												
59	do	173	29	22	Mar. 31, 1930	3¾	4.5	101.3	4	3,950	0	0	Average.
60	do	111	(?)	103	Nov. 28, 1929	6	7.5	102.4	3	5,150	×	×	Do.
61	do	116	14	84	Dec. 7, 1929	5	5	104	8	2,950	×	×	Severe.
62	do	110	23	75	Nov. 28, 1929	4.5	6.5	103.2	6	2,600	×	0	Average.
63													
64	*Aëdes ægypti*	117	17	58	Dec. 10, 1929	6	4.5	102.2	6	2,600	×	0	Do.
65	do	112	27	10	Dec. 10, 1929	6	5.5	102.4	7	3,150	×	×	Severe.
66	do	114	24	109	Dec. 10, 1929	5	4	101.4	3	4,150	×	×	Average.
67	do	137	32	89	Jan. 27, 1930	5	6	103	5	2,900	×	×	Do.
68	do	139	41	110	Jan. 27, 1930	5.5	5.5	103.8	5	3,400	×	×	Do.

[c] Hormone broth, 7 cubic centimeters; 1.5 cubic centimeters given, Berk. "V."

TABLE 14.—*Experimental dengue in man*—Continued.

Infection of volunteers.						Incubation period in volunteers.	Character of infection.						Character of attack.
Volunteer No.	Infected mosquitoes used.						Fever.		Leukopenia.		Rash.		
	Species.	Lot No.	Age of virus.	Number fed or injected.	Date fed or injected.		Duration.	Maximum temperature.	Duration.	Minimum count.	Primary.	Secondary.	
			Days.			*Days.*	*Days.*		*Days.*				
69	*Aëdes albopictus*	128	56	14	Jan. 27, 1930	5	5.5	102.6	8	3,150	0	×	Average.
70	*Aëdes ægypti*	137	52	7	Feb. 6, 1930	5	5	102.8	4	3,150	×	0	Do.
71	do	137	52	6	Feb. 6, 1930	6	2	102.6	7	2,900	0	×	Mild.
72	do	125		11									
		126	(?)	56	Jan. 27, 1930	4	5	104	4	3,100	0	×	Average.
		145		65									
73													
74	*Aëdes ægypti*	135	17	46	Jan. 27, 1930	5.5	3.5	102.4	6	3,100	0	×	Do.
75	do	138	47	60	Feb. 3, 1930	5	5.5	103.8	7	2,150	×	×	Do.
76	do	137	46	61	Feb. 1, 1930	6	3.5	101.2	7	3,300	0	×	Mild.
77	do	162	15	87	Feb. 17, 1930	4.5	6.5	102	7	1,750	0	×	Average.
78	do	138	57	26	Feb. 12, 1930	4.5	5	103.8	7	3,050	0	×	Do.
79	*Aëdes albopictus*	160	22	118	Feb. 24, 1930	5	5	103.6	0	0	×	×	Do.
80	*Aëdes ægypti*	156	21–25	73	Feb. 15, 1930	6	4.5	102	4	3,450	0	×	Do.
81	do	157	22–26	87	Feb. 15, 1930	5.5	4.5	102.4	6	1,950	0	×	Do.
82	do	167	19	108	Mar. 4, 1930	4.5	6.5	101.4	3	4,100	0	×	Do.
83	do	168	19	11	Mar. 8, 1930	5	5	102.6	5	2,600	×	0	Do.
84	do	137	70	21	Feb. 24, 1930	4.5	8	102.4	6	3,200	0	×	Do.
85	do	164	52	5	Mar. 26, 1930	6.25	5.5	101.8	4	2,800	0	0	Do.
86	do	165	46	23	Mar. 26, 1930	5.25	5	103.6	7	2,400	0	×	Do.

87	do	159	49	3	Mar. 22, 1930	9.25	6	104.2	6	2,700	0	0	Do.
88	do	178 181	17 15	28 19	Mar. 22, 1930	5	3.5	103.2	2	3,200	×	×	Mild.
89	do	171	37	15	Mar. 31, 1930	5.25	4.25	103.6	7	1,900	0	×	Average.
90	*Aëdes albopictus*	183	18	15	Apr. 1, 1930	4.5	5.25	102.4	6	2,900	×	×	Do.
91	*Aëdes ægypti*	174	29	21	Apr. 1, 1930	5.25	4.5	103.8	6	4,400	0	×	Do.
92	do	189	(?)	73	Apr. 10, 1930	(?)	3.5	102.4	3	3,600	0	×	Do.
93	*Aëdes albopictus*	183	19	9	Apr. 1, 1930	9.25	1.5	101.4	6	3,600	0	×	Mild.
94	*Aëdes ægypti*	189	0	125–150	Mar. 24, 1930	7–9	5	104.6	6	3,100	0	×	Average.

The blood.—While routine erythrocyte counts were not made, no remarkable change was observed in the size, shape, color, or staining reactions of the red cells in specimens prepared daily for differential leukocyte counts. In two cases daily counts were made of the blood platelets throughout the periods of observation, incubation, and fever, but no significant variation from normal was observed.

The leukopenia and other leukocytic reactions associated with dengue are considered to be the most significant diagnostic signs of the disease. Leukopenia was observed at some time during each of the experimental infections, and after its initial appearance it was progressive, often lasting for several days after the subsidence of the fever. Because of the great importance of the leukocytic reactions to the clinical diagnosis of dengue, this subject is discussed in detail elsewhere in this report; however, it may be stated here that leukopenia, accompanied by the so-called "shift to the left" of the granulocytes, with evidence of degeneration of the mature granulocytes was observed in all of the uncomplicated cases of dengue considered in this study.

SUMMARY

The analysis of the clinical aspects of dengue fever, based on experimental cases, the majority of which were transmitted by mosquitoes, may be summarized as follows:

The average incubation period was 5.66 days; average duration of fever, 4.8 days; with primary rash, 38.8 per cent; with secondary rash, 69.5 per cent. Additional symptoms commonly observed were leukopenia, 100 per cent; postorbital pains and backache, 90 per cent; altered sense of taste, 65 per cent; pains in limbs, 60 per cent; adenopathy, 47 per cent; and pains in joints, 43 per cent.

The character of the infection in eighty-one cases may be classified as mild, 13.6 per cent; average, 70.4 per cent; and severe, 16 per cent. The onset was abrupt, usually with chill or chilliness, in 58 per cent of sixty cases considered.

It is believed that passage of the virus in series through a number of human beings and mosquitoes did not alter the virulence of the organism; that the number of mosquitoes used to transmit dengue to man, within the limits of one to one hundred thirty-nine, did not influence the character of the infection; that variations in the character of the attacks noted were probably due to individual differences in the susceptibility of the volunteers infected.

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NUMERICAL AND MORPHOLOGICAL ALTERATIONS OF THE LEUKOCYTES DURING DENGUE FEVER

It has long been recognized that typical dengue fever in man is accompanied by a characteristic decrease in the leukocytes of the blood. Attention was called to the diagnostic significance of changes in the leukocytes during dengue by Carpenter and Sutton,(1) who, in 1905, observed that a leukopenia, "with a normal differential leukocytic count or varying degrees of small mononuclear lymphocytosis and a strongly marked eosinophilia late in the disease is characteristic of dengue, and that convalescence is almost invariably ushered in by a pronounced mononuclear lymphocytosis which persists for several days. These observers found the average total leukocytic counts to be about 3,500, with individual variations between a high of 5,866 and a low of 1,866. Stitt,(2) in 1907, who differentiated dengue from influenza and other acute infections by means of leukocyte counts, observed that a progressive leukopenia occurred in dengue and that the total counts varied between 5,280 and 1,450. From differential counts made at different periods of the disease, he found that marked variations occurred, there being at first a large increase in small lymphocytes succeeded by a greater increase in large lymphocytes and finally by an increase in large mononuclear cells.

Ashburn and Craig,(3) in 1907, discussed the leukopenia of dengue and presented results obtained by Vedder, who made the following remarks concerning his work:

> The polymorphonuclear leukocytes are greatly decreased and the small lymphocytes are greatly increased, while the large lymphocytes are moderately increased during the latter days of the illness. These changes are to a greater or less degree, characteristic of the disease throughout its duration. The decrease in polymorphonuclears and the increase in small lymphocytes takes place almost at once, being very noticeable on the second day of illness.

From these and other observations on experimental cases, Ashburn and Craig concluded that—

> Dengue is characterized by a leukopenia and in the vast majority of instances by a decrease in the polymorphonuclear leukocytes and a marked

increase in the small lymphocytes; the increase in the small lymphocytes is constant throughout the disease.

Many later workers have commented on the leukopenia of dengue, and several studies have been made of experimentally induced infections. One of the most important of these studies was made by Siler, Hall, and Hitchens(4) (1926) who analyzed one hundred fifty-four leukocyte counts of blood specimens from forty-eight experimental cases of dengue, or an average of over three counts per case. Their conclusions were as follows:

> The leukocyte count is not a dependable early symptom, as counts taken within a few hours of onset show great variation and average about normal. With the progress of the disease the total leukocyte count is reduced, the mean for the first four days becoming progressively less. However, 25 per cent of our cases failed to show a leukopenia at any time during the course of the disease.
>
> The so-called "reversal" in the differential count, the reduction of the percentage of polynuclear neutrophiles and corresponding increase of lymphocytes, is shown to be a function of the total count, both in normal and in dengue cases. Dengue cases showing normal total counts do not show the differential change. The differential count of the white cells is thus seen to be of little diagnostic value in comparison with the total count.
>
> The degree of leukopenia appears to be independent of the temperature at the time the count was made, and also of the severity of the disease as judged by the highest temperature reached during the attack, or by the duration of fever.

In 1929 there appeared a report by Joannides(5) concerning the morphologic alterations in the leukocytes during dengue, which is of considerable interest in connection with the present study. During the dengue pandemic in Athens in 1928, Joannides examined the blood of patients on different days of their illness, using smear preparations fixed with alcohol and stained by Giemsa's method. He noted especially profound morphological alterations both in the large mononuclears and the neutrophilic polymorphonuclear cells.

PRESENT INVESTIGATION

The following analysis of the leukocyte changes associated with dengue is based on total and differential leukocyte counts made on eighty-one experimental American volunteers. Throughout the various investigations of dengue carried on by the board, it was a routine procedure to examine the blood of each volunteer daily, in order to make the diagnosis in the experimental infections more certain. In practically all cases the counts were made at 6.30 a. m., before breakfast, and the afternoon counts were usually made between 4 and 4.30 p. m.

THE LEUKOCYTES OF NORMAL INDIVIDUALS

Volunteers admitted to the hospital for the various dengue experiments were generally observed for a preliminary period of eight or nine days, in order to obviate the possibility of outside infections. In many instances leukocyte counts were made daily throughout the preliminary period of normal observation. The results of these observations, which are summarized in Table 15 and fig. 21, furnish normal figures for comparison with those obtained during and following dengue fever.

THE LEUKOCYTES DURING SEVEN DAYS PRECEDING THE ONSET OF DENGUE FEVER

As shown in Table 15 and fig. 21, the average leukocyte counts during a period of seven days prior to the onset of dengue

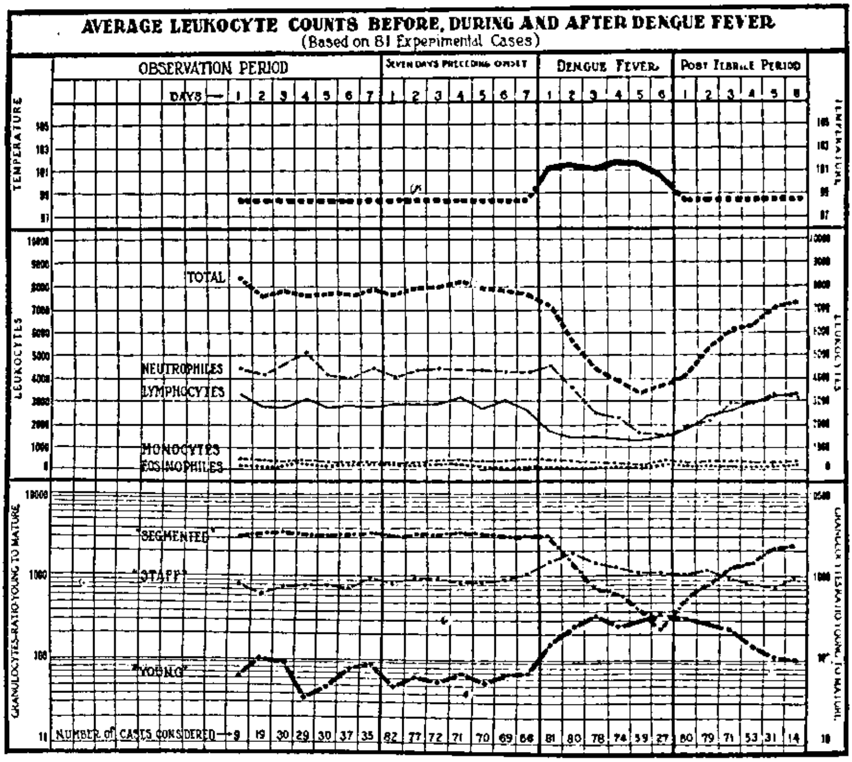

FIG. 21. Average leukocyte counts before, during, and after dengue fever.

fever were essentially similar to those noted in the normal observation period, except that on the day preceding fever the average figures indicate an alteration in the proportion of immature to mature forms of the granulocytes.

THE LEUKOCYTES DURING DIFFERENT DAYS OF EXPERIMENTALLY INDUCED DENGUE FEVER; TOTAL NUMBERS OF LEUKOCYTES

In the cases considered in Table 15 and fig. 21, the average total leukocyte counts were normal during the first day of the fever. However, as will be seen by reference to the charts in the Appendix, in some cases there was a slight leukopenia at the beginning of the initial rise in temperature, while in others there was a definite increase of short duration in leukocytes. In general, the leukopenia began on the second day of fever and progressed daily, reaching an average low point of 3,386 in about five days after onset. Thereafter the return of the leukocytes to normal was gradual, and required a period of from three to five days after the temperature had become normal.

Leukopenia occurred in every case at some time during the febrile period, with two exceptions. One of these patients had furunculosis and the other acute bronchitis. However, in the former there was a relative decrease in leukocytes, and in the latter definite leukopenia occurred during the post-febrile period.

The results shown in Table 16 indicate the proportion of cases in which one may expect to find leukopenia both during and following fever. Although all cases had leukopenia at some time during the fever, it will be noted that this sign was found more constantly toward the close of the febrile period and was constant for a few days following the last day of fever. The results emphasize the fact that in any case suspected as dengue, the blood should be examined on several successive days before it can be concluded that leukopenia does not exist.

The relationship between the fevers of different durations and the average total leukocyte counts is indicated in Tables 17, 18, 19, 20, and 21, and text figs. 22, 23, 24, 25, and 26.

It will be noted that in cases with fever lasting only two days, the leukopenia usually began on the second day. The average low point reached was 4,437 cells on the second day after the fever had subsided; and the leukopenia persisted in the average throughout the six-day postfebrile period. The total period of leukopenia in these two-day fever cases was about seven days.

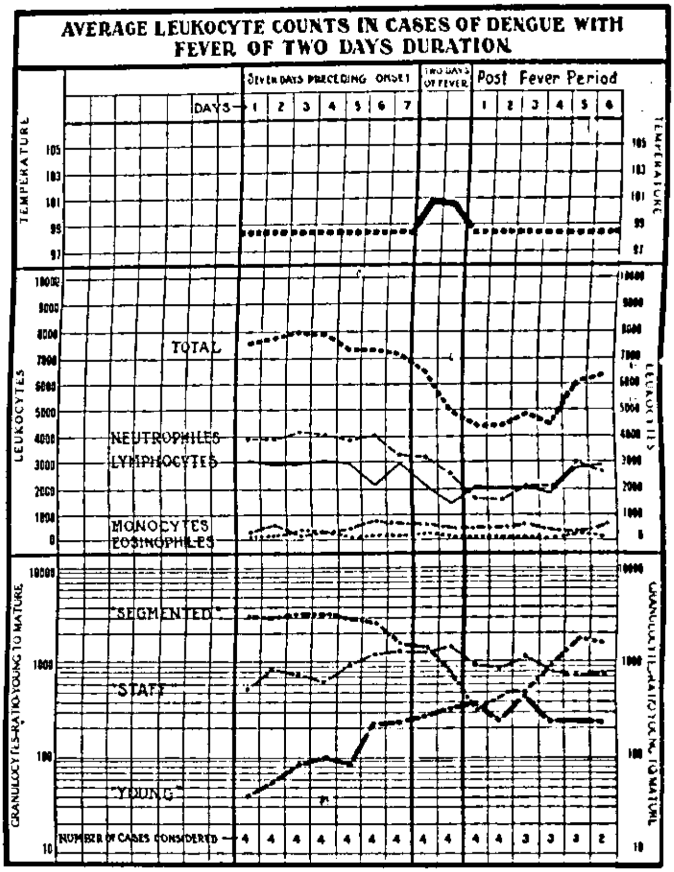

FIG. 22. Average leukocyte counts in cases of dengue with fever of two days duration.

In a small group of patients who had fever for three days, the average curve showed leukopenia on the second and third days, reaching an average low point of 4,375 on the first day of normal temperature, with a return to normal on the third day after subsidence of the fever. The average total period of leukopenia was four days.

In the cases with fever lasting four days, the average leukocyte curve showed a progressive fall beginning on the second day of fever and reaching a low point of about 3,520 on the first day of normal temperature; after which the counts rose daily until they were normal about the fourth day following the fever. The total average period of leukopenia in this four-day-fever group was about six days.

TABLE 15.—*Average leukocyte counts before, during, and after dengue fever.*

Days.	Cases considered.	Temperature.	Leukocyte counts.								
			Pulse.	Total.	Granulocytes.					Lymphocytes.	Monocytes.
					Neutrophilic.				Eosinophilic.		
					Segmented.	Staff.	Young.	Total.			
NORMAL OBSERVATION.											
1	9	98.4	84	8,255	3,016	840	61	4,292	247	3,222	433
2	19	98.1	81	7,681	3,494	650	112	4,108	126	2,843	364
3	30	98.1	79	7,850	3,686	731	94	4,768	218	2,827	309
4	29	97.8	80	7,634	3,262	788	35	5,136	231	3,029	306
5	30	98.1	80	7,773	3,286	793	47	4,133	216	2,855	336
6	37	97.9	77	7,655	3,263	740	76	4,068	238	2,915	379
7	35	98.2	80	7,929	3,588	949	84	4,473	256	2,814	337
Average ----		98.1	80	7,825	3,371	784	72	4,425	219	2,929	352
SEVEN DAYS PRECEDING ONSET OF DENGUE.											
7	66	97.8	78	7,746	3,143	880	49	4,097	226	2,981	376
6	69	97.9	78	7,946	3,353	928	62	3,369	229	2,960	360
5	70	97.8	78	8,016	3,363	936	57	4,445	235	2,983	376
4	71	97.8	77	8,165	3,420	879	67	4,357	259	3,104	402
3	72	98.0	79	7,937	3,417	859	54	4,342	212	2,854	375
2	77	97.8	79	7,821	3,169	911	65	4,196	211	3,007	366
1	82	97.8	79	7,711	3,146	1,116	70	4,267	237	2,779	398
Average ----		97.9	78	7,906	3,287	930	61	4,282	231	2,953	379
FEVER PERIOD.											
1	83	101.1	84	7,129	3,046	1,608	181	4,607	182	1,818	416
2	82	101.3	90	5,617	1,556	1,903	280	3,661	64	1,475	372
3	80	101.2	88	4,569	787	1,602	301	2,663	58	1,537	340
4	77	101.5	87	4,035	629	1,392	283	2,322	53	1,369	271
5	62	101.4	86	3,886	425	1,127	299	1,873	58	1,345	217
6	29	100.6	86	3,800	374	1,162	351	1,887	58	1,588	287
Average ----		101.1	87	4,756	1,136	1,469	283	2,836	79	1,522	317
POSTFEVER PERIOD.											
1	80	98.0	75	4,314	554	1,061	321	1,949	113	1,959	379
2	79	97.8	76	5,469	787	1,119	261	2,369	182	2,454	407
3	71	97.8	77	6,195	1,274	963	243	2,803	133	2,660	461
4	53	97.7	77	6,441	1,499	809	155	2,848	142	2,918	415
5	31	98.0	75	7,103	2,191	775	128	3,343	182	3,194	426
6	14	97.8	79	7,219	2,273	939	116	3,165	207	3,148	415
Average ----		97.9	76	6,123	1,429	944	204	2,746	159	2,722	417

TABLE 16.—*Leukopenia on the different days of dengue.*[a]

DENGUE FEVER.

Day.	Cases considered.	Leukopenia.		No leukopenia.	
		Number.	Per cent.	Number.	Per cent.
1	82	24	29.27	58	70.73
2	81	53	65.43	28	34.57
3	79	69	87.34	10	12.66
4	75	71	94.68	4	5.34
5	60	57	95.00	3	5.00
6	28	26	92.85	2	7.15
FOLLOWING RETURN OF TEMPERATURE TO NORMAL.					
1	80	72	90.00	8	10.00
2	79	58	73.42	21	26.58
3	71	31	43.66	40	56.34
4	53	16	30.19	37	69.81
5	32	9	28.12	23	71.88
6	14	3	21.43	11	78.57

[a] Leukopenia occurred in all the cases at some time during the fever.

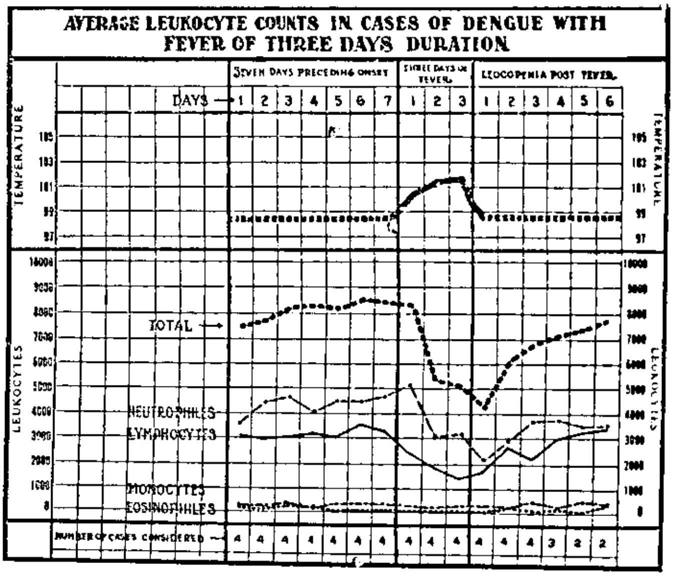

FIG. 23. Average leukocyte counts in cases of dengue with fever of three days duration.

TABLE 17.—*Average leukocyte counts in cases of dengue with fever of two days duration.*

SEVEN DAYS PRECEDING FEVER.

Days.	Cases considered.	Temperature.	Leukocyte counts.							
			Total.	Granulocytes.					Lymphocytes.	Monocytes.
				Neutrophilic.				Eosinophilic.		
				Segmented.	Staff.	Young.	Total.			
7	4	(a)	7,662	3,229	553	40	3,997	272	3,035	483
6	4	(a)	7,800	3,113	806	55	3,974	297	2,947	578
5	4	(a)	7,962	3,452	730	81	4,263	402	2,966	299
4	4	(a)	7,825	3,393	638	97	4,127	345	3,082	328
3	4	(a)	7,325	2,939	936	83	3,819	164	2,929	467
2	4	(a)	7,312	2,626	1,238	223	4,089	233	2,239	720
1	4	(a)	7,175	1,800	1,259	242	3,301	186	2,952	663
(Maximum)[b]			9,300	4,092	2,123	616	5,544	1,215	4,071	1,078
(Minimum)[b]			6,700	1,035	365	75	1,725	69	1,001	79
Average			7,580	2,936	880	117	3,939	271	2,879	505
FEVER PERIOD.										
1	4	100.9	6,539	1,664	1,256	277	3,198	313	2,129	602
2	4	100.6	4,950	799	1,540	342	2,681	164	1,526	520
(Maximum)[b]		102.2	7,200	2,849	2,548	476	4,134	486	2,592	1,248
(Minimum)[b]		97.2	4,300	44	616	208	924	0	583	301
Average		100.8	5,744	1,231	1,390	309	2,939	238	1,827	561
POSTFEVER PERIOD.										
1	4	(a)	4,462	326	932	364	1,623	116	2,137	540
2	4	(a)	4,437	443	872	250	1,565	179	2,069	506
3	3	(a)	4,850	508	1,014	485	2,008	94	2,070	611
4	3	(a)	4,550	984	797	238	2,020	158	1,856	418
5	3	(a)	6,150	1,706	727	225	2,958	205	2,775	404
6	2	(a)	6,375	1,632	765	229	2,626	192	2,818	510
(Maximum)[b]			7,650	2,448	1,336	627	3,901	307	3,900	969
(Minimum)[b]			2,900	153	434	0	1,274	58	841	186
Average			5,137	933	851	299	2,133	157	2,287	498

[a] Normal.

[b] Not averages.

TABLE 18.—*Average leukocyte counts in cases of dengue with fever of three days duration.*

Days.	Cases considered.	Temperature.	Leukocyte counts.				
			Total.	Granulocytes.		Lymphocytes.	Monocytes.
				Neutrophilic.	Eosinophilic.		
SEVEN DAYS PRECEDING FEVER.							
7	4	([a])	7,525	3,720	238	3,104	352
6	4	([a])	7,862	4,403	175	2,910	348
5	4	([a])	8,287	4,716	245	3,068	394
4	4	([a])	8,350	4,018	275	3,316	190
3	4	([a])	8,200	4,548	61	3,023	245
2	4	([a])	8,562	4,468	75	3,686	395
1	4	([a])	8,475	4,782	134	3,391	368
(Maximum)[c]			12,100	7,744	605	5,568	968
(Minimum)[c]			6,450	2,819	0	2,092	161
Average			8,179	4,379	172	3,214	327
FEVER PERIOD.							
1	4	100.7	8,237	5,243	51	2,570	329
2	4	101.3	5,375	3,072	31	1,989	268
3	4	101.6	5,025	3,150	25	1,545	303
(Maximum)[c]		104	11,600	9,512	137	3,699	472
(Minimum)[c]		99	3,150	1,549	0	1,116	126
Average		101.2	6,212	3,822	36	2,035	100
POSTFEVER PERIOD.							
1	4	([a])	4,375	2,202	34	1,821	267
2	4	([a])	6,262	2,980	255	2,739	232
3	4	([a])	6,812	3,779	58	2,282	441
4	3	([a])	7,117	3,733	94	2,957	259
5	2	([a])	7,425	3,560	74	3,269	483
6	2	([a])	7,750	3,551	306	3,434	418
(Maximum)[c]			8,100	4,785	476	4,131	670
(Minimum)[c]			3,700	1,599	0	1,406	123
Average			6,624	6,301	137	2,750	350

[a] Normal.

[b] No Schiling counts made.

[c] Not averages.

TABLE 19.—*Average leukocyte counts in cases of dengue with fever of four days duration.*

SEVEN DAYS PROCEEDING FEVER.

Days.	Cases considered.	Temperature.	Leukocyte counts.								
			Total.	Granulocytes.						Lymphocytes.	Monocytes.
				Neutrophilic.				Eosinophilic.			
				Segmented.	Staff.	Young.	Total.				
7	12	(a)	8,196	3,424	839	22	4,295	291		3,285	326
6	12	(a)	8,229	3,575	877	69	4,506	195		3,091	384
5	12	(a)	8,400	3,670	738	67	4,475	248		2,304	428
4	12	(a)	8,829	3,904	743	71	4,666	284		3,245	506
3	12	(a)	7,950	3,427	748	46	4,157	218		3,088	374
2	13	(a)	8,373	3,339	811	93	4,222	271		3,301	514
1	14	(a)	7,557	3,197	982	73	4,197	202		2,670	464
(Maximum)[b]			12,300	5,600	3,198	369	7,749	1,069		4,669	1,674
(Minimum)[b]			5,900	649	268	0	3,127	0		1,485	0
Average			8,219	3,505	818	63	4,360	244		2,998	428
FEVER PERIOD.											
1	14	100.9	7,007	2,878	1,567	202	4,640	192		1,667	452
2	13	101	5,912	1,919	1,669	251	3,624	80		1,631	533
3	15	101.2	4,963	942	1,477	391	2,646	47		1,604	503
4	15	101.4	4,517	715	1,416	322	2,550	69		1,468	387
(Maximum)[b]		104	9,650	6,552	2,832	686	7,720	786		2,848	1,914
(Minimum)[b]		99.2	3,100	158	341	52	1,094	0		606	32
Average		101.1	5,600	1,613	1,532	291	3,385	97		1,592	469
POSTFEVER PERIOD.											
1	15	(a)	3,520	409	1,103	280	1,815	153		1,810	391
2	14	(a)	5,132	700	1,145	340	2,171	161		2,336	395
3	14	(a)	6,093	1,269	1,028	161	2,574	164		2,915	360
4	10	(a)	6,575	1,271	893	146	2,529	173		3,331	476
5	7	(a)	7,678	2,819	610	95	3,579	361		3,328	537
6	4	(a)	7,987	3,342	721	58	4,121	202		3,148	459
(Maximum)[b]			10,900	8,612	1,782	819	4,505	517		5,014	1,668
(Minimum)[b]			2,700	162	380	51	792	36		1,056	38
Average			6,164	1,634	917	180	2,798	202		2,811	436

[a] Normal.

[b] Not averages.

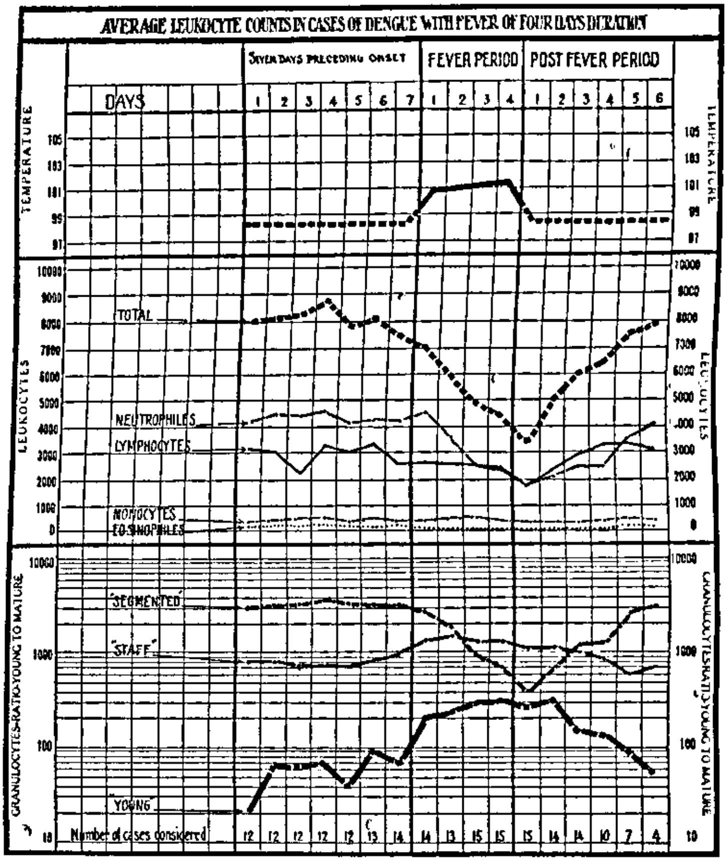

FIG. 24. Average leukocyte counts in cases of dengue with fever of four days duration.

The curve for the five-day-fever group also showed that leukopenia began on the second day, progressed daily, and reached a minimum point of 3,172 on the fifth day of fever. From this point the average counts increased, reaching normal four days later. The average duration of leukopenia in this group of cases was six days.

The cases with fever lasting six days showed a very similar average leukocyte curve during the period of fever, with the leukopenia persisting for two days afterward, or for a total of about seven days.

From a consideration of all these groups it may be stated that leukopenia was always apparent by the second day of fever; and that, although the lowest average counts in the groups of cases with short febrile periods were not quite so low, the

TABLE 20.—*Average leukocyte counts in cases of dengue with fever of five days duration.*

SEVEN DAYS PRECEDING FEVER.

Days.	Cases considered.	Temperature.	Leukocyte counts.							
			Total.	Granulocytes.					Lymphocytes.	Monocytes.
				Neutrophilic.				Eosinophilic.		
				Segmented.	Staff.	Young.	Total.			
7	22	(a)	8,261	3,346	965	79	4,410	261	3,132	410
6	25	(a)	7,880	3,399	847	65	4,211	211	3,009	332
5	25	(a)	7,830	3,309	812	19	4,209	243	2,970	387
4	26	(a)	8,010	3,558	803	58	4,324	251	2,900	404
3	27	(a)	8,191	3,552	922	48	4,578	231	2,940	411
2	29	(a)	7,595	3,190	807	48	4,137	227	2,868	337
1	31	(a)	7,931	3,410	1,091	59	4,352	305	2,680	457
(Maximum)[b]			11,150	5,555	4,263	334	8,251	930	5,329	1,069
(Minimum)[b]			5,950	307	298	0	1,836	0	1,540	0
Average			7,957	3,395	892	54	4,317	247	2,927	391
FEVER PERIOD.										
1	32	101.7	7,146	3,225	1,607	185	4,838	165	1,577	451
2	32	101.4	5,723	1,627	1,851	334	3,623	43	1,474	447
3	32	100.9	4,395	822	1,430	292	2,592	87	1,426	407
4	32	102	3,919	740	1,292	300	2,033	51	1,275	253
5	32	101.2	3,172	447	1,076	301	1,912	30	1,054	208
(Maximum)[b]		104.4	9,900	5,247	4,717	819	7,209	1,089	3,857	1,384
(Minimum)[b]		98.4	1,900	76	351	0	646	0	328	0
Average		101.4	4,871	1,372	1,451	282	2,999	75	1,361	333
POSTFEVER PERIOD.										
1	31	(a)	4,374	525	950	355	1,912	155	1,869	352
2	31	(a)	5,661	959	1,120	252	2,524	143	2,563	512
3	27	(a)	6,505	1,603	855	146	2,744	150	3,024	462
4	20	(a)	7,055	1,660	628	114	2,711	145	3,311	516
5	9	(a)	7,211	2,145	762	96	3,295	136	3,377	409
6	3	(a)	7,650	2,905	688	115	3,670	203	3,213	495
(Maximum)[b]			8,700	3,268	1,848	891	5,046	655	4,514	1,192
(Minimum)[b]			2,150	117	196	0	539	0	881	0
Average			6,409	1,633	834	179	2,809	155	2,893	458

[a] Normal.

[b] Not averages.

minimum was reached, as in the fevers of long duration, on the fourth or fifth day after onset; and that the duration of leukopenia in all groups was about four to seven days. This fact indicates that regardless of the duration of fever the decrease

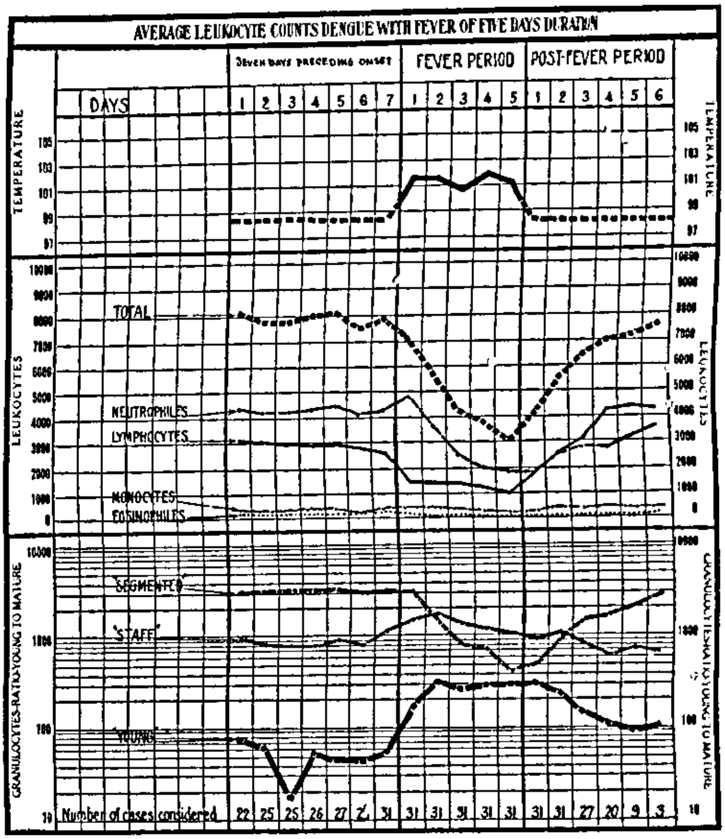

FIG. 25. Average leukocyte counts in cases of dengue with fever of five days duration.

in the number of leukocytes, once begun, progresses to a low point which occurs on the fourth, fifth, or sixth day, and that thereafter the number gradually increases and returns to normal. Apparently, differences in the length of the febrile period do not influence to any marked extent either the degree or duration of leukopenia.

THE LYMPHOCYTES

The average daily counts of the numbers of lymphocytes in the various groups of cases considered showed the following general trend (Table 15): During the periods of normal observation and incubation, the average number of cells varied between 2,827 and 3,222, with an average of about 2,900. On the day preceding onset of dengue the lymphocytes were slightly decreased, while on the first day of dengue fever the lymphocytes

TABLE 21.—*Average leukocyte counts in cases of dengue with fever of six days duration.*

Days.	Cases considered.	Temperature.	Leukocyte counts.							
			Total.	Granulocytes.					Lymphocytes.	Monocytes.
				Neutrophilic.				Eosinophilic.		
				Segmented.	Staff.	Young.	Total.			
SEVEN DAYS PRECEDING FEVER.										
7	23	(a)	7,720	3,776	978	40	3,984	193	2,898	380
6	24	(a)	7,935	3,170	1,059	68	4,375	256	2,935	344
5	25	(a)	7,978	3,162	1,172	68	4,456	221	2,887	332
4	25	(a)	8,032	3,018	1,065	67	4,216	261	3,198	374
3	25	(a)	7,672	3,186	880	46	4,202	232	2,867	330
2	27	(a)	7,689	3,054	1,006	88	4,075	187	2,997	276
1	28	(a)	7,552	3,026	1,183	54	4,283	198	2,724	266
(Maximum)[b]			13,300	6,765	5,586	294	8,778	940	4,980	1,200
(Minimum)[b]			3,900	195	246	0	1,448	0	1,410	0
Average			7,797	3,199	1,049	54	4,227	221	2,929	329
FEVER PERIOD.										
1	27	101.4	7,181	2,840	1,574	118	4,622	173	2,061	315
2	27	102.0	5,605	1,452	2,219	218	3,924	74	1,241	275
3	27	101.0	4,656	715	1,839	213	2,724	27	1,632	280
4	27	101.2	3,852	450	1,448	223	1,913	51	1,404	206
5	27	101.5	3,509	390	1,208	274	1,825	91	1,278	232
6	27	100.6	3,713	227	1,224	339	1,902	52	1,486	276
(Maximum)[b]		104.6	9,800	5,947	4,588	1,512	7,278	749	3,952	1,228
(Minimum)[b]		98.6	2,100	28	370	0	798	0	380	0
Average			4,758	1,012	1,585	231	2,818	78	1,517	264
POSTFEVER PERIOD.										
1	27	(a)	4,837	656	1,218	307	2,193	105	2,159	344
2	26	(a)	5,854	1,051	1,291	204	2,607	171	2,564	392
3	23	(a)	6,717	1,716	955	183	2,908	188	3,008	432
4	17	(a)	6,906	2,127	919	124	3,246	140	3,133	409
5	10	(a)	7,050	2,095	1,001	97	3,321	134	3,222	298
6	3	(a)	6,333	1,212	1,584	63	2,859	132	3,127	194
(Maximum)[b]			8,800	5,104	2,660	657	6,160	602	4,917	1,341
(Minimum)[b]			2,150	43	441	0	530	0	957	0
Average			6,283	1,809	2,161	163	2,856	145	2,869	345

[a] Normal. [b] Not averages.

were usually decreased about 1,000 cells. Some decrease was maintained throughout the febrile period with an average low point of 1,522 cells, or approximately one-half the average nor-

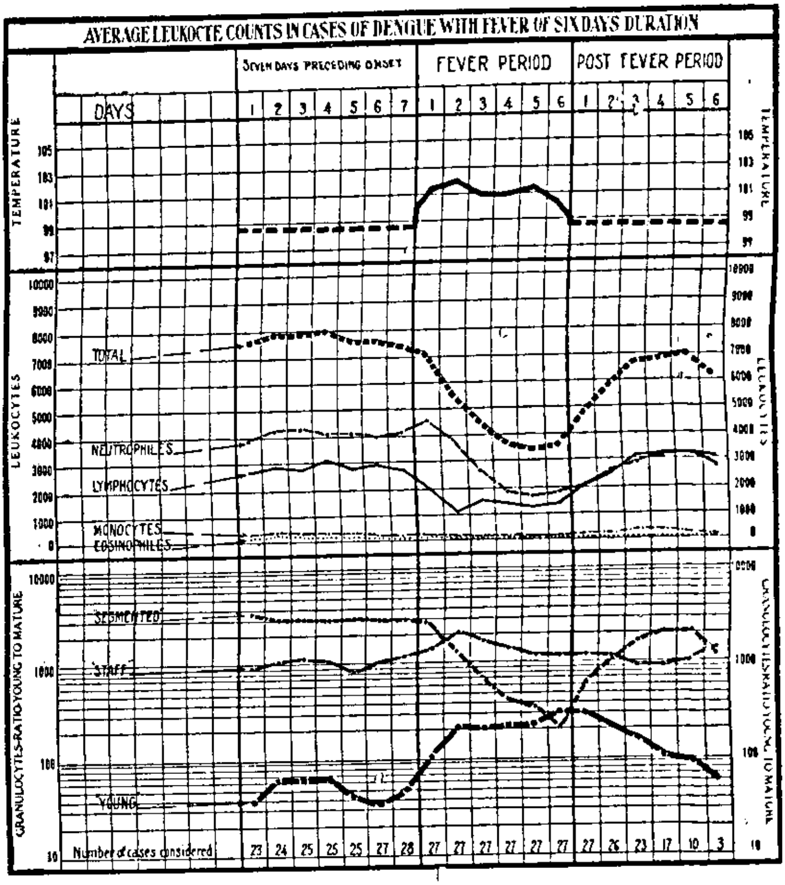

FIG. 26. Average leukocyte counts in cases of dengue with fever of six days duration.

mal number. Although the average reduction in the number of lymphocytes in the eighty-one cases considered extended over a period of nine days, the charts of the individual fever groups show that this period might be as short as six days, but as a general rule the lymphocytes returned to normal before the granulocytes.

THE MONOCYTES

The average daily counts of monocytes during the period of normal observation and incubation varied from a minimum of 306 to a maximum of 433, with an average of 365 (Table 15). While no significant changes were noted in the total numbers of the monocytes during dengue, a large vacuolated mononuclear cell, morphologically similar to the so-called Turk "irritation cell," made its appearance in the majority of cases either during the last days of fever or at some time in the period of convalescence. This type of cell has been noted by various observers in other febrile conditions including Tsutsugamuchi disease, in which Kawamura(6) observed that "Turk" cells usually appeared late, or at the time when the eruption was fading. Although the finding of these cells was not considered as diagnostic, their occurrence came to be regarded as suggestive of dengue.

THE GRANULOCYTES (EOSINOPHILIC)

The average daily count of the eosinophiles before the onset of fever was 231. For the period of fever the average was 79 and in the postfebrile period it was 159 (Table 15). Because of the small numbers of the eosinophiles present, these changes may be of no significance, but it is interesting to note that the eosinophiles apparently follow the general downward trend of the other granulocytes.

THE GRANULOCYTES (BASOPHILIC)

The basophiles showed no noticeable change and were omitted from the charts.

THE GRANULOCYTES (NEUTROPHILIC)

The most significant diagnostic leukocytic reactions associated with dengue fever are the profound changes in the neutrophilic granulocytes. It will be noted that the average daily neutrophilic curves parallel those of the total leukocytes.

The average daily numbers of neutrophiles during the periods of normal observation and incubation varied from 3,369 to 5,136, with an average for both groups of about 4,300. During the first day of dengue fever the average showed a slight increase. This was followed on the second day of fever by a sharp decrease of about 1,000 cells. Thereafter, the decline was progressive until a low point below 2,000 was reached at some time between the fourth and sixth days following onset of dengue, regardless of the duration of the fever. Following this low point the numbers of neutrophiles slowly increased daily; but in a majority of the groups, and in the average of all groups, they were still slightly below normal even on the sixth day of convalescence.

In addition to a marked decrease in numbers during dengue, the mature neutrophilic granulocytes also showed morphologic evidence of degenerative changes. During the course of the disease, especially during the period of extreme leukopenia, it was not infrequently noted that the nuclei of these cells were markedly altered; in many cells they were vacuolated, irregular in outline, and poorly stained; in some they were fragmented, portions of nuclear material being found irregularly distributed in the cytoplasm. The cytoplasm of the cells often showed a marked loss of neutrophilic granules with some clumping of those remaining. The outlines of these cells were usually indefinite and appeared to fade into surrounding areas. Because of the marked destruction of the circulating granulocytes, one should expect to find some compensating reaction on the part of the blood-forming organs. This reaction is shown by a study of the granulocytes, which were arbitrarily divided into three groups as follows:

"Segmented" neutrophiles.—These cells, which are considered to be the mature forms of the granulocytes, are characterized by a multilobed nucleus segmented or divided into two or more lobes connected by a thread of nuclear material.

"Staff" or "band" form neutrophiles.—These cells are considered to be younger than the segmented forms. They have a nonsegmented nucleus which is narrow and usually curved. The cytoplasmic structure is similar to that of the fully matured cells.

"Young" granulocytes or metamyelocytes.—These cells are considered to be more immature than the "staff" form and slightly older than the myelocytes. The nucleus is usually rounded in outline and often shows slight indentations. The cytoplasmic structures are similar to those of the mature cells.

It is apparent from a study of the charts and tables, that the leukopenia of dengue was due largely to a destruction of the mature granulocytes. These cells actually decreased from a normal level of approximately 3,000 to the alarmingly low figure of about 300 cells. Fortunately, this loss of mature granulocytes was partially compensated for by an immediate increase in the immature forms, which doubled or tripled in numbers during the febrile period.

The mature, or "segmented," forms usually remained at the normal level of about 3,000 cells until the second day of the fever, when there was a fall of about 50 per cent, following which the numbers of cells continued to decrease to a low point of about 300 to 400 on the fifth or the sixth day following the onset. After this low point was reached the numbers of mature granulocytes gradually increased toward normal during convalescence, but in the average of all cases and in some of the groups the normal level was not reached even on the sixth day following normal temperature.

In the majority of the fever groups, the "staff," or immature, forms increased to a point above the upper normal limit of 1,000 cells on the day preceding the febrile period, but the rise shown by the average of all cases was insignificant. In general, it may be stated that these cells doubled in numbers on the first day of fever, which no doubt accounted for the slight elevation observed in the total neutrophilic count on the first day. On the second day of fever the average number of staff forms reached a peak of about 1,900 cells, which also sustained the total count to a level only slightly below normal. From this high point the staff forms remained increased in number during fever and for three or four days following the return of temperature to normal.

The "young"-form granulocytes, the upper normal limit of which was considered to be about 100 cells, showed a sharp rise on the first day of fever and were more than doubled in numbers by the second day. At the period of greatest depression of the mature forms the young granulocytes were still increased in numbers and in the majority of the four groups they later became more numerous than the former. The highest point was usually reached between the fifth and sixth days of fever; following which they remained above normal for about three more days in the different fever groups, while the average of all cases showed an increase in these cells during the entire six days of the postfebrile period.

From a consideration of the changes in the proportions of the three types of granulocytes, it may be stated that an increase in the number of immature cells, associated with a normal number or a slight decrease in the number of mature forms, during the first day of a febrile condition clinically resembling dengue, is a valuable diagnostic sign. In many individual cases this reaction appeared on the day preceding the onset and made it possible to forecast a diagnosis of dengue in these experimental cases.

In cases with fever, leukopenia, and subjective symptoms of dengue the increase in immature granulocytes above the numbers

DENGUE IMMUNITY

There is an abundance of epidemiological and clinical information bearing on the subject of dengue immunity, but relatively few experimental studies have been reported. Therefore, many questions remain to be answered concerning the mechanism, degree, and duration of this immunity. From observations concerning the high percentage of individuals attacked during the first introduction of dengue into various localities, and even in endemic areas during epidemic or pandemic waves of the disease, it seems probable that few if any human beings possess natural immunity to the infection. Outbreaks have been reported by Sandwith,(1) Godding,(2) Cleland, Bradley, and McDonald,(3) Legendre,(4) and others, in which from 75 to 100 per cent of entire populations have been infected within a limited period of time. A recent example is the explosive outbreak in Greece during 1927–1928, in which, according to a report by the League of Nations,(5) 239,000 cases occurred in Athens during the first eight months of 1928.

While adult natives living in endemic localities rarely become ill with the disease during inter-epidemic periods, there is no conclusive evidence to indicate that natural immunity to dengue exists in any group or race of people. The apparent racial immunity of certain peoples living in the Tropics is, no doubt, acquired as a consequence of previous attacks. According to Hirsch,(6) (1883) it was observed by Dickson that the only persons who escaped infection during the Charleston outbreak of 1850 were those who had previously been attacked in 1828. A most significant observation is that of Bonne,(7) (1918) in Paramaribo, who noted that Europeans admitted to his hospital developed dengue within a week or two; while no case occurred among one hundred Creole nurses and other attendants. However, he observed that the mulatto children were not immune. Other interesting observations have been made in the Fiji Islands. Skottowe(8) (1890) reported that during 1885, when dengue was probably first introduced, the native Fiji Islanders suffered more severely than the Melanesians. Later, during an epidemic which occurred there in 1906, Montague(9) observed that the old residents who had had the disease in 1885 escaped in almost every instance, and that in the intermission, the island had been particularly free from dengue. During the recent epidemic in Greece it was noted by Copanaris(10) (1928) that persons who had had the disease in 1927 escaped in 1928. It was remarked that "presumably some immunity had been produced and remained." Blanc and Caminopetros(11) (1929) made similar observations concerning the immunity of individuals who had dengue during the 1927 outbreak in Greece, and stated that, in their opinion, dengue confers a high degree of immunity which is of considerable duration.

Adult natives living in endemic areas of the Philippines are also comparatively resistant to dengue, as is indicated both by the observations of local physicians and by a comparison of the low dengue rates for Philippine Scout soldiers who have spent most of their lives in the infested areas, with the relatively high rates for white American troops stationed for short periods in the same localities. This fact was discussed by Siler, Hall, and Hitchens,(12) who made the following observations:

> In an area of endemicity it is likely that native children suffer their first attack of dengue early in life, and that the degree of immunity exhibited later in life is the result of acquired immunity constantly reënforced by frequent bites of infective mosquitoes. In every epidemic season a certain number of native people have clinical dengue. These may be persons who develop immunity with difficulty or who return to the susceptible state rapidly, or who have been removed from infective mosquitoes for a considerable period.

The fact that many adult natives of endemic areas are immune is also indicated by the results of transmission experiments. Ashburn and Craig(13) 1907, after the inoculation of fourteen volunteers at Fort William McKinley, observed that among seven men who had not previously been exposed, only one immune was found; while among seven individuals who had passed through an epidemic, two were completely immune, three were partially immune, and in one the result was doubtful. Siler, Hall, and Hitchens,(12) during their transmission experiments, were able to infect all but seven of sixty American volunteers inoculated with infective blood or bitten by infective mosquitoes. All but one of the seven immunes had been in the Philippines for periods of from three to nineteen months. However, in their experiments with thirty-two Philippine Scout soldiers from Fort William McKinley it was found that 100 per cent were immune. Schule(14) 1928 called attention to the fact that certain volunteers inoculated with dengue virus were immune and that all of these immunes had either been residents of the Philippines for a considerable period or had come from other localities where dengue was prevalent.

Somewhat similar results were obtained during the investigations conducted by the present board. Among eighty-seven American volunteers who were bitten by mosquitoes of infective lots only 4, or 4.6 per cent, failed to develop dengue. These four immunes had been in the Philippines for periods of three, six, six, and eight months, respectively, before they were tested. Similar experiments with Philippine Scouts stationed in or near Manila gave strikingly different results which are described below.

PART I. OBSERVATIONS CONCERNING THE IMMUNITY OF NATIVES OF THE PHILIPPINE ISLANDS

(A) ATTEMPTS TO PRODUCE DENGUE IN ADULT FILIPINOS LIVING IN AREAS IN WHICH THE DISEASE IS ENDEMIC

Among fifteen Philippine Scout soldiers, each inoculated with blood from experimental cases of dengue, and eighteen others bitten by infective lots of *Aëdes*, only two showed suspicious but unproved signs of infection; while thirty-one, or 93.9 per cent, were immune. In each experiment the soldiers were observed daily for evidence of infection, the temperature reactions were recorded and leukocyte counts were made each morning, in order to detect even slight changes which might indicate infection in individuals incompletely immune.

Experiment 1.—January 12, 1929. Infective blood, which had been collected in citrate from an experimental case of dengue (A.V. 1-H), thirty-six hours after onset of symptoms, and kept in the ice box for twenty-four hours, was inoculated, in 0.025 cubic centimeter amounts, into each of ten Philippine Scout soldiers stationed at Fort William McKinley. During subsequent observation none of these men developed symptoms recognizable as dengue. The records of their daily temperature reactions and leukocyte counts are shown below.

Filipino volunteer 1.—Pilorin, D., Serial No. 673082, Ilocano, born in Ilocos Sur, age 22, service in Army four years at Fort William McKinley. Has never been sick except for "chills and fever" in 1928. "Never had dengue or 'trankaso,' or any symptoms suggesting dengue." The highest temperature recorded for this man was 99° F. which occurred January 17, 1928, the fifth day after inoculation. The only leukocyte count low enough to indicate a mild leukopenia was 6,150 January 16, the fourth day after inoculation. These observations were not considered to be of diagnostic significance.

Filipino volunteer 2.—Dona, Alejandro, Serial No. R-310178, Ilocano, born in Ilocos Sur, age 27, service in Army eleven years at Fort William McKinley. Had "chills and fever" when a child, but has no knowledge of any attacks of "trankaso" or dengue. After inoculation the only elevation of temperature noted was 99 and 99.8° F. on the eighth day, January 20; the leukocyte counts January 16, 21, and 23 were 5,200, 6,250, and 6,100, respectively.

Filipino volunteer 3.—Robio, Catalino, Serial No. R-323307, Ilocano, born in Ilocos Norte, age 28, service in Army eleven years, including one

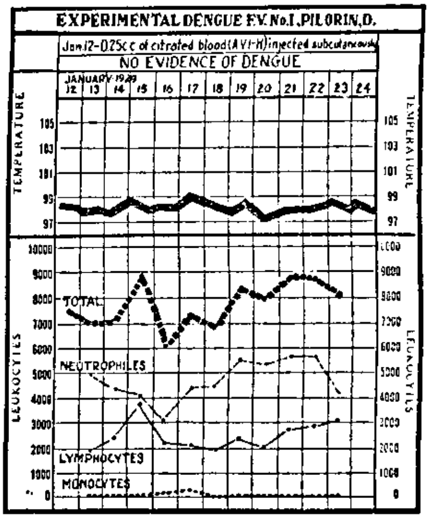

FIG. 27. Experimental dengue in Filipino volunteer 1.

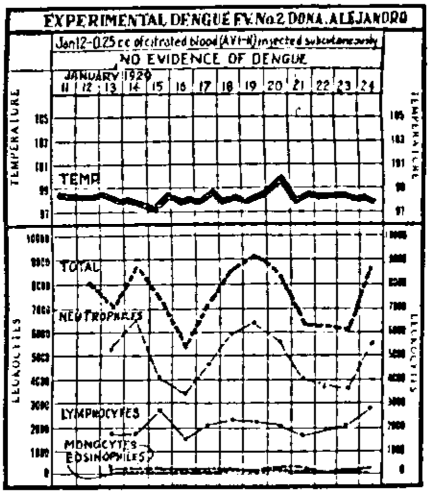

FIG. 28. Experimental dengue in Filipino volunteer 2.

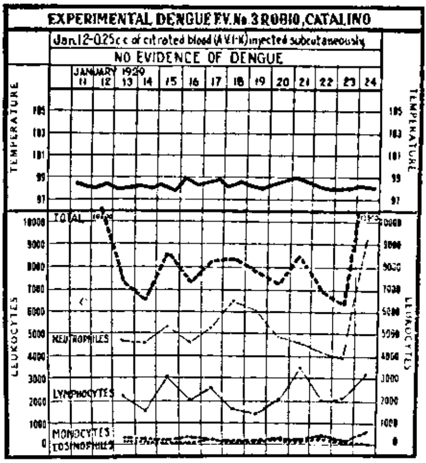

FIG. 29. Experimental dengue in Filipino volunteer 3.

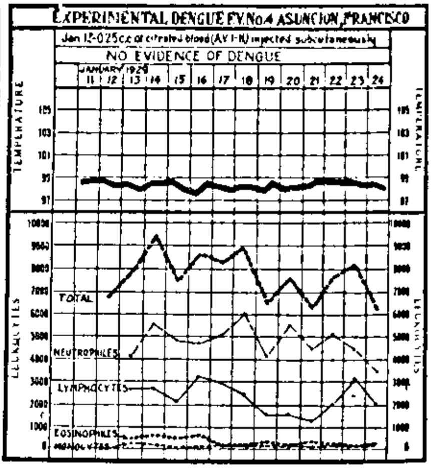

FIG. 30. Experimental dengue in Filipino volunteer 4.

year in Batangas and nine years in Fort William McKinley. Has never been sick. After inoculation the temperature was normal for twelve days and there was no significant reduction in leukocytes, with the possible exception of counts of 6,500 January 14 and 6,250 January 23, which were not considered diagnostic.

Filipino volunteer 4.—Asuncion, Francisco, Sgt., Serial No. 6736039, Ilocano, born in Abra, age 24, service in Army four years at Fort William McKinley. Had fever during childhood but has not been ill since he became grown. After inoculation his temperature was normal for twelve days. The leukocyte count was 6,500 January 19, 6,350 January 21, and 6,150 January 24. There was no evidence of dengue.

Filipino volunteer 5.—Laureta, Agustin, Serial No. R-321311, Ilocano, born in Abra, age 32, ten years service in Army at Fort William McKinley. "Never sick during childhood." Had "trankaso" in 1919 but "never had dengue." After inoculation with dengue blood the temperature remained normal. The leukocyte count was 13,000 on the second day, 6,600 on the sixth, and 5,700 on the eleventh. There was no evidence of dengue.

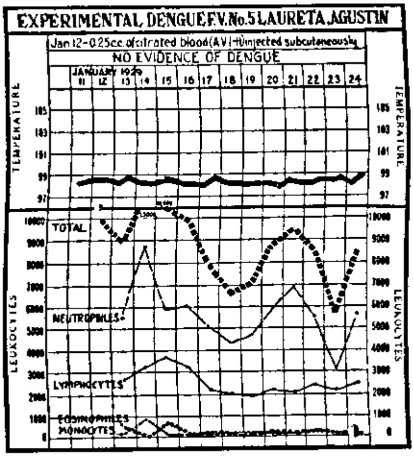

Fig. 31. Experimental dengue in Filipino volunteer 5.

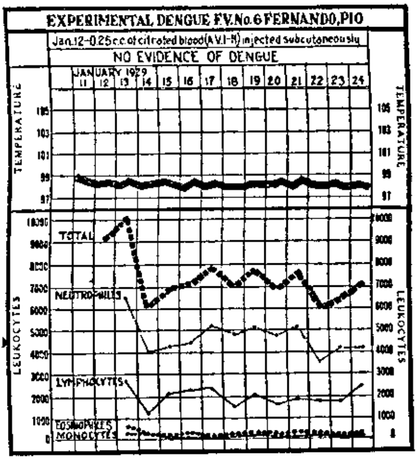

Fig. 32. Experimental dengue in Filipino volunteer 6.

Filipino volunteer 6.—Fernando, Pio, Cpl., Serial No. R-310605, Ilocano, born in Ilocos Sur, age 40, service in Army 21$\frac{8}{12}$ years (Lanao, 1913–1916; Camp McGrath, 1916–1919; Fort William McKinley, 1919–1929). Had fever during childhood, also malaria in 1915. Never had "trankaso" or dengue. After inoculation the temperature remained normal. The leukocyte count was 6,050 on the second day, 6,100 on the tenth day, and 6,500 on the eleventh day. There was no evidence of dengue.

Filipino volunteer 7.—Fontanilla, Jose, Serial No. 6736088, Ilocano, born in Ilocos Sur, age 24, service in Army four years at Fort William McKinley. Never sick during childhood, but had "trankaso" in 1918. After inoculation the temperature and total leukocyte counts were normal or slightly elevated for twelve days. There was no indication of dongue.

Filipino volunteer 8.—Balosan, Domingo, Serial No. R-321024, Ilocano, born in Abra, age 33, service in Army ten years at Fort William McKinley. Had "fever with aching of eyes and joints" during childhood and had "fever in 1918." After inoculation the temperature remained normal for twelve days and the lowest leukocyte count obtained was 5,450 on the fourth day. There was no evidence of dengue.

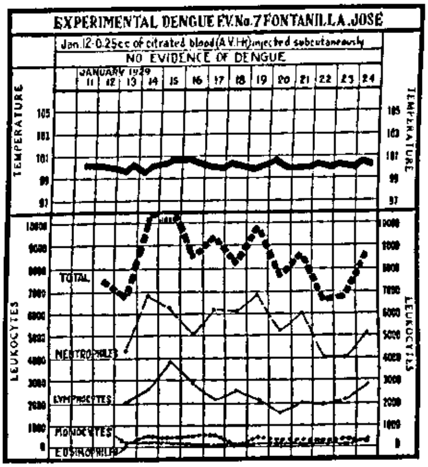

FIG. 33. Experimental dengue in Filipino volunteer 7.

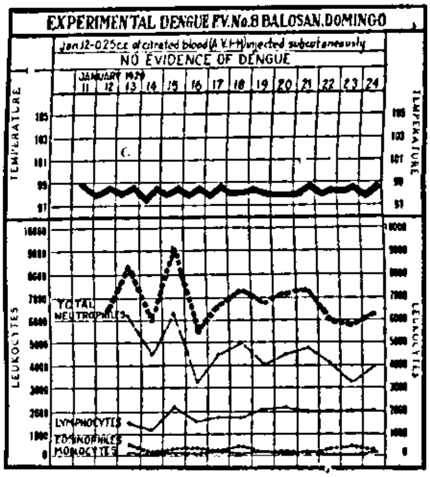

FIG. 34. Experimental dengue in Filipino volunteer 8.

Filipino volunteer 9.—Reyno, Pascual, soldier on duty at Fort William McKinley. No history of dengue. After inoculation the temperature remained normal and the leukocyte counts were decreased slightly after the fifth day. However, there was no evidence of dengue.

Filipino volunteer 10.—Abad, Jose, Serial No. 6610113, Tagalog, born in Nueva Ecija, age 27, lived in Cabanatuan, Nueva Ecija. Service in Army nine years at Fort William McKinley. Had "fever" when a child; also had "chills and fever" in 1917. This volunteer was injected with 0.5 cubic centimeter of infective dengue virus in February, 1925, by Siler, Hall, and Hitchens, but showed no reaction. After the present inoculation the temperature remained normal for twelve days, as did the total leukocyte count, except for a decrease to 5,850 on the fourth day. The leukocyte curve in this man was not considered significant of dengue, but was thought to be suggestive.

Experiment 2.—Filipino volunteer 12, Sales, Gervacio, Ilocano, born in Ilocos Norte, age 32, service in Army nine years, Fort Mills and United States Army Medical Department Research Board in Manila. Had "fever" during childhood, also "chills and fever" in 1924.

February 9, 1929, Sales was bitten by sixty-one infective *Aëdes ægypti*, lot 2, which had taken infective blood thirty-one days previously from an experimental case of dengue (A.V. 2-M) during the first day of the disease. These mosquitoes produced dengue in a susceptible American volunteer (A.V. 4-Y).

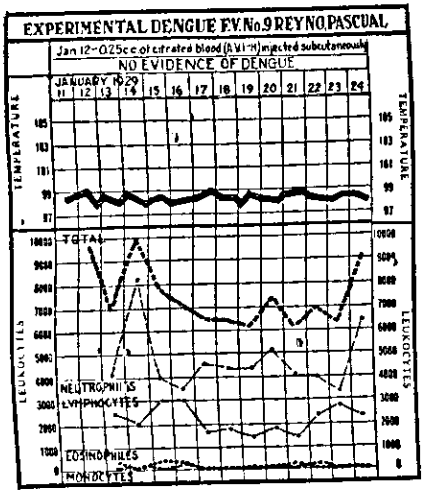

FIG. 35. Experimental dengue in Filipino volunteer 9.

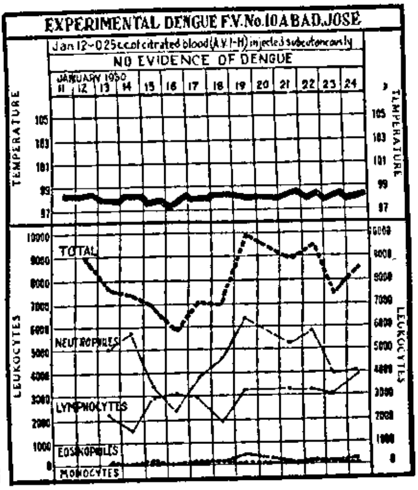

FIG. 36. Experimental dengue in Filipino volunteer 10.

After being bitten by the mosquitoes the temperature of F.V. 12–Sales, remained normal for twelve days, excepting a rise to 99.2° F. on the fifth day, at which time the leukocyte count was 6,850. There was no evidence of dengue. Note: This man was used later in experiment 5.

Experiment 3.—Filipino volunteer 11, Reyes, Bonifacio, Pangasinan, born in Pangasinan, age 31, service in Army seven years ten months, at United States Army Medical Department Research Board, Manila. During childhood had chicken pox, small pox, chills and fever; also had fever which he thinks was "dengue," with aching of eyes, bones, and joints, in 1919, 1924, and 1928.

February 9, 1929, was bitten by fifty-six infective *A. ægypti*, lot 3, which thirty days previously had taken blood from an experimental case of dengue (A.V. 1–H) during the first day of the disease. These mosquitoes transmitted dengue to a susceptible American volunteer (A.V. 3–A). After he had been bitten by the infective mosquitoes, Reyes's temperature and lymphocyte counts remained normal for at least twelve days. Note: This man was used later in experiment 6.

INOCULATION OF FILIPINOS WITH LARGE AMOUNTS OF DENGUE VIRUS IN AN ATTEMPT TO OVERCOME THEIR IMMUNITY

Experiment 4.—April 16, 1929. Blood collected in sodium citrate from an experimental case of dengue (A.V. 6–R) on the first day of the disease, was injected into three Filipino Scout soldiers as follows:

Filipino volunteer 17.—Yap, A., was injected subcutaneously with 5 cubic centimeters of the blood. This man, who was a private 1st class in the 12th Medical Regiment, was born in Leyte, age 31, had had six years service in the Army at Fort William McKinley. Previous medical history and physical examinations were negative. Three days after the inoculation of infective blood Yap was admitted to hospital complaining of headache and pain in his bones and joints. The temperature war 101.6° F. on this day. The total leukocyte counts on the fourth and fifth days were 5,250 and 5,350, respectively, and there was an indefinite rash over his body on the day of admission. Comment: Symptoms which might be ascribed to a mild attack of dengue were observed in this Filipino after the subcutaneous inoculation of 5 cubic centimeters of infective blood, and while the specific nature of the infection was not proved, it is believed that dengue occurred.

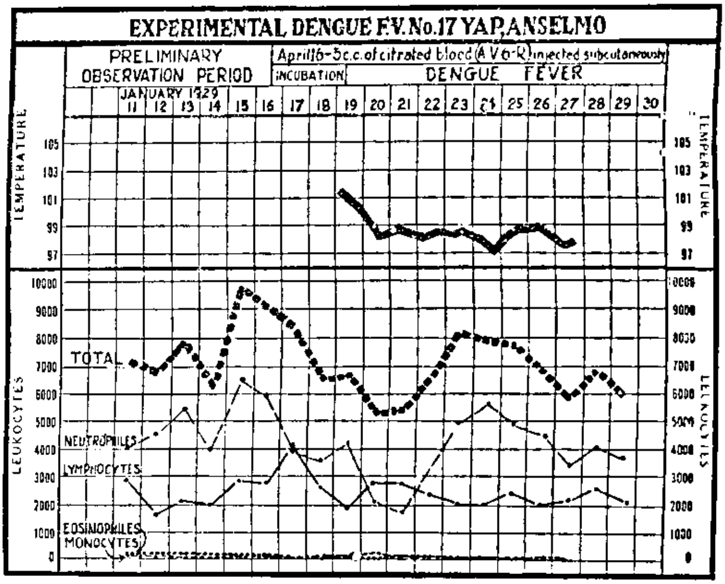

FIG. 37. Experimental dengue in Filipino volunteer 17.

Filipino volunteer 18.—Penas, Eleodoro, was injected intravenously with 0.5 cubic centimeter of the infective blood April 16. This man, who was a private in the 12th Medical Regiment, was born in Camarines Sur, age 26, service in Army five years at Fort William McKinley. Previous medical history and physical examination negative. Three days after the inoculation of blood, he was admitted to hospital complaining of headache, pains in eyes and joints. The temperature was elevated on the fourth, fifth, and sixth days. The only suggestive leukocyte counts were 5,250 on the seventh day and 6,050 on the eighth. Comment: This case, like the preceding one, presented symptoms which suggested the possibility of a mild dengue infection.

Filipino volunteer 19.—De los Santos, F., was inoculated subcutaneously with 8 cubic centimeters of the infected blood April 16. This man, who was a private in the 12th Medical Regiment, stationed at Fort William McKinley, was observed for a period of eleven days, during which he remained well and had normal temperature reactions and leukocyte counts. There was no indication of dengue.

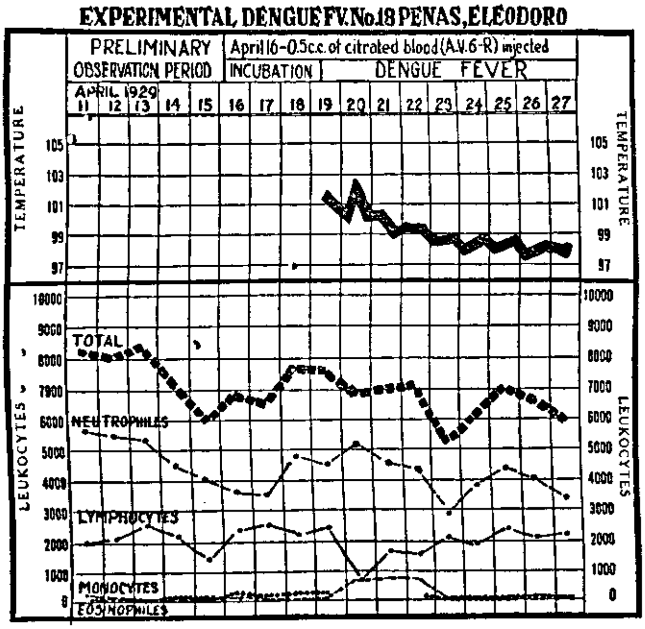

FIG. 38. Experimental dengue in Filipino volunteer 18.

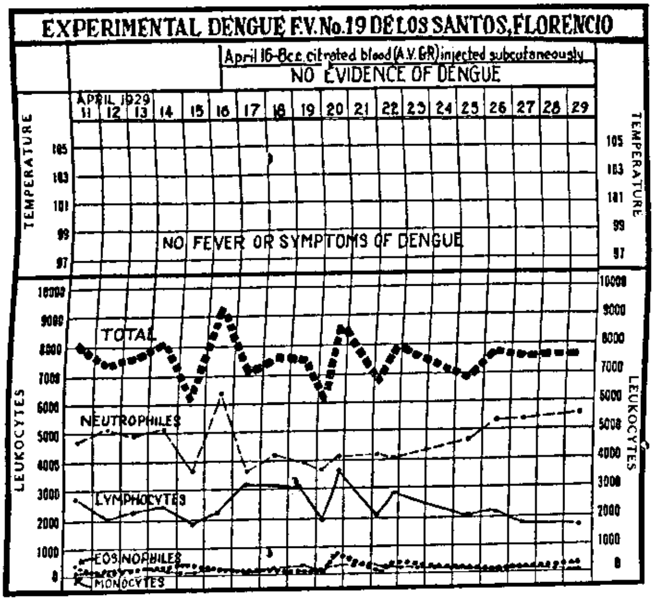

FIG. 39. Experimental dengue in Filipino volunteer 19.

AN ATTEMPT TO PRODUCE DENGUE IN AN IMMUNE VOLUNTEER BY REPEATED FEEDINGS OF DENGUE-INFECTED MOSQUITOES

Experiment 5.—Filipino volunteer 12, Sales, used for this experiment was a technician in the laboratory of the board. So far as he knew he had never had dengue, but stated that he had "fever" during childhood. Since 1928, two negative attempts were made to infect Sales with dengue; one (*a*) February 9, 1929, when he was bitten by sixty-one dengue-infected *A. ægypti* of lot 2, and the other (*b*) in 1929, when he was injected subcutaneously with citrated infective blood from an experimental case of dengue. The third attempt was made to overcome the resistance of this individual by first allowing two infective mosquitoes to feed on him and, later, introducing a larger dose of virus by allowing a large number of infected mosquitoes to feed.

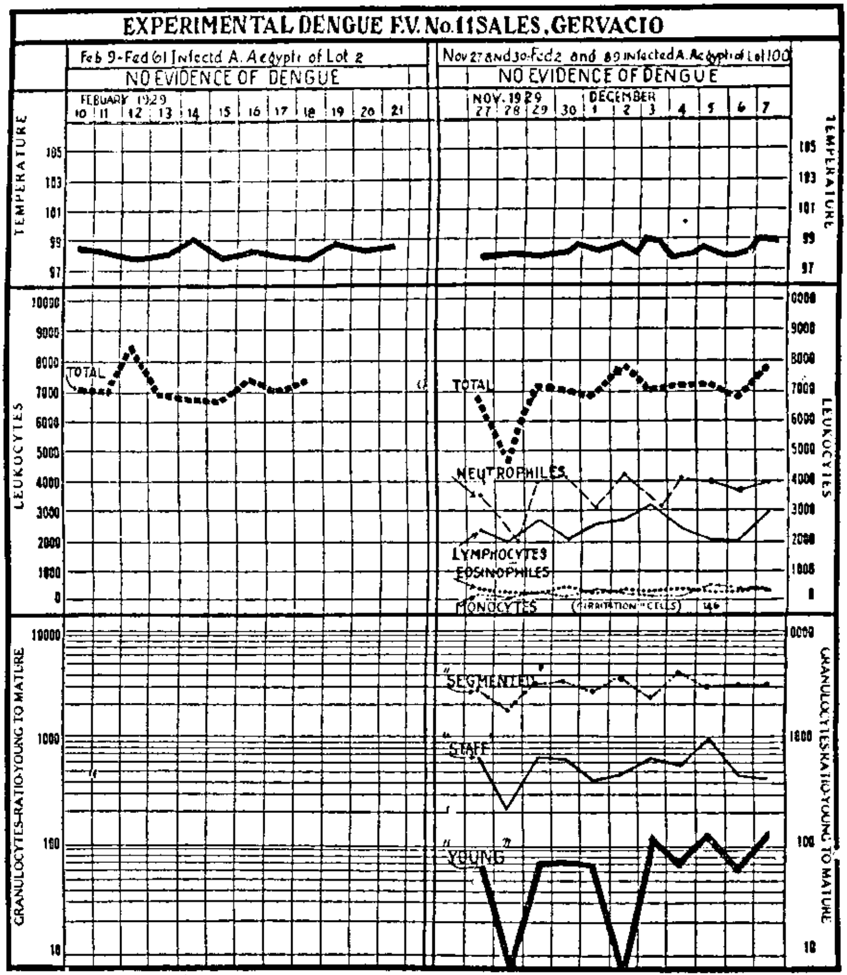

FIG. 40. Experimental dengue in Filipino volunteer 11.

November 27 Sales was bitten by two infective *A. ægypti*, lot 100, which had taken blood 27 days previously from an experimental case of dengue (A.V. 55–S), during the first day of the disease. November 30, he was bitten by eighty-nine infective *A. ægypti*, lot 100. Temperature and leukocyte count records made from November 27 to December 7, 1929, indicate that this man failed to develop even a mild infection. Comment: This adult Filipino was found to be immune to repeated doses of dengue virus.

AN ATTEMPT TO PRODUCE DENGUE IN AN IMMUNE FILIPINO VOLUNTEER BY REPEATED DAILY FEEDINGS OF INFECTIVE MOSQUITOES

Experiment 6.—Filipino volunteer 11. Reyes, selected for this experiment, was a laboratory technician who for six years had assisted in handling the mosquitoes used by the board in various dengue transmission experiments. Pvt. Reyes stated that he had had dengue fever several years previously.

Since 1928 two negative attempts were made to infect Reyes with dengue: (*a*) February 9, 1929, he was bitten by fifty-six dengue-infected *A. ægypti*, lot 3, and (*b*) later in 1929, he was inoculated subcutaneously with citrated infective blood from an experimental case of dengue. He remained well following both tests.

Present test November 19, 1929, 9.40 a. m. Bitten by ten infective *A. ægypti* of lot 104, which had fed eighteen days previously on an experimental case (A.V. 55–S), during the first day of dengue fever; temperature 98°, leukocyte counts normal (7,400). November 20, 8 a. m., bitten by nine *A. ægypti*, lot 104; temperature 98.2°, leukocytes 6,800. November 21, 8 a. m., bitten by nine *A. ægypti*, lot 104; temperature 98°, leukocytes 8,100. November 22, 8 a. m., bitten by ten *A. ægypti*, lot 104; temperature 98.4°, leukocytes 7,350. November 23, 8 a. m., bitten by thirty-five *A. ægypti*, lot 104; temperature 98°, leukocytes 8,100. November 24, 8 a. m., bitten by nine infective *A. ægypti*, lot 100, which had fed twenty-four days previously on an experimental case (A.V. 55–S), during the first day of dengue; temperature 98.4°, leukocytes 6,800. November 25, 8 a. m., bitten by ten *A. ægypti*, lot 100; temperature 97.9°, leukocytes increased to 11,200. November 26, 8 a. m., bitten by ten *A. ægypti*, lot 100; temperature 97.3°, leukocytes 10,650. November 27, 8 a. m., bitten by eight *A. ægypti*, lot 100; temperature 97.7°, leukocytes 9,900. Following this ninth feeding of infective mosquitoes, the volunteer remained well for at least five months. There was no elevation of temperature and no leukopenia during ten days following the last biting. Comment: It is apparent that this man possessed a strong protective immunity against infection with dengue.

EXPERIMENTS TO DETERMINE THE POSSIBILITY OF PRODUCING SYMPTOMLESS DENGUE IN APPARENTLY IMMUNE PHILIPPINE SCOUT SOLDIERS LIVING IN ENDEMIC AREAS

Experiment 7.—March 25, 1929. Four volunteers, F.V. 13, Aguinaldo, Vicente; F.V. 14, Novilla, Filomeno; F.V. 15, Villeza, Cipriano; and F.V. 16, Villarin, Candido, were admitted to the experimental ward at Sternberg Hospital. The following day (March 26) they were each bitten by ten infective *A. ægypti* of lots 9 and 10. As shown by their clinical records, including daily temperature and leukocyte counts, there was no clinical evidence of dengue during the periods of subsequent observation.

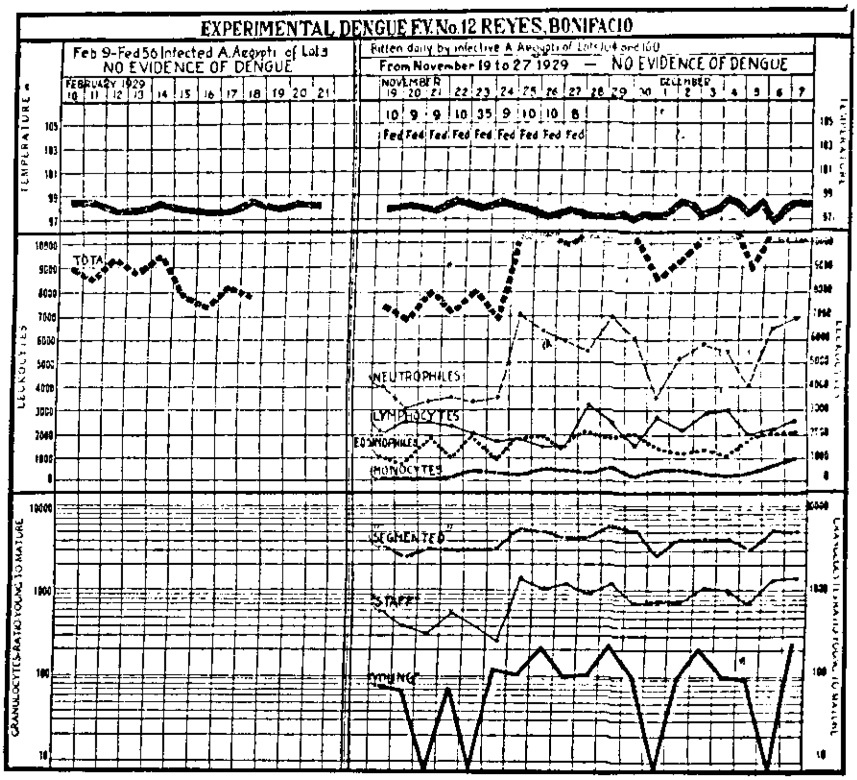

FIG. 41. Experimental dengue in Filipino volunteer 12.

(*a*) *Filipino volunteer 13.*—Aguinaldo, Serial No. 6612777, Ilocano, born in Ilocos Norte, age 29, service in Army six years at Fort William McKinley. He denied ever having had dengue or other diseases. However, an old hospital chart shows that he at one time had a positive Wassermann reaction and had received treatment. At present there is no indication of syphilis. Intestinal parasites were found in the fæces. March 25, 1929, he was bitten by seventy-nine *A. ægypti*, of lot 10; and, as indicated by the subsequent temperature reactions and leukocyte counts, there was no clinical evidence of infection.

(*b*) *Filipino volunteer 14.*—Novilla, Filomeno, Pvt., Co. "E," 57th Infantry, born in Malag, Capiz, age 24, service in Army six months at Fort William McKinley. Had "trankaso" during childhood but states that he has had no other illness. Physical examination and laboratory tests showed him to be normal. March 25, 1929, bitten by one hundred twenty-three dengue-infective *A. ægypti* of lot 10. Both the temperature reaction and leukocyte counts remained normal for ten days.

(*c*) *Filipino volunteer 15.*—Villeza, Cipriano, Pvt., 35th Ambulance Co., 12th Medical Rgt., born in Lubao, Pampanga, age 26, service in Army seven years at Fort William McKinley. Denied having had dengue or other diseases, except operation for stone in bladder or kidney. Physical examination at present time showed no abnormality, except intestinal parasites, glandular enlargement, and a doubtfully positive Wassermann reaction. March 25, 1929, he was bitten by fifty-eight dengue-infective *A. ægypti* of lot 9, after which the temperature and leukocyte counts remained normal for at least ten days.

(*d*) *Filipino volunteer 16.*—Villarin, Candido, Pvt., Co. "E," 57th Infantry, born in Dumarao, Capiz, age 28, service in Army nine years at Fort William McKinley. Previous medical history negative, except for arthritis in 1926. Present examination negative, except for intestinal parasites. March 26, 1929, he was bitten by thirty-seven dengue-infective *A. ægypti* of lot 9, after which both temperature and leukocyte counts remained normal for at least ten days.

DAILY TESTS FOR VIRUS IN THE BLOOD OF FILIPINO VOLUNTEERS 13, 14, AND 16 (EXPERIMENT 7) FOR TEN DAYS AFTER THEY WERE BITTEN BY INFECTIVE AËDES.

Test 1.—One cubic centimeter of blood was collected in citrate every day from each of the three Filipino volunteers. March 29 the blood specimens collected during the first three days were pooled and passed through a preliminary type of Mandler filter, after which 0.5 cubic centimeter was injected subcutaneously into a susceptible American volunteer (A.V. 5–M). Dengue did not develop.

Test 2.—Blood specimens collected from each of the three Filipinos on the fourth, fifth, and sixth days after they were bitten by infective mosquitoes were pooled, filtered, and injected into a susceptible American volunteer (A.V. 6–R) with similar negative results.

Test 3.—Blood collected from each of the Filipinos on the seventh, eighth, ninth, and tenth days was filtered and injected into an American volunteer (A.V. 7–M). He was later shown to be immune.

Test 4.—Normal *A. ægypti* were fed on each of the three Filipino volunteers each day throughout the experiment. April 16 all of the one-, two-, and three-day mosquitoes were combined and called lot 13; and eighty-three fed on a susceptible American volunteer (A.V. 8–G). Dengue did not develop.

Test 5.—April 20, ninety-three *A. ægypti* of the combined groups designated lot 14, which had taken blood from the Filipinos on the fourth, fifth, and sixth days, fed on a susceptible American volunteer (A.V. 9–D) with similar negative results.

Test 6.—April 23, one hundred sixty *A. ægypti* of the combined lots designated as lot 15, which had taken blood from the Filipinos on the seventh, eighth, ninth, and tenth days, bit a susceptible American volunteer (A.V. 12–S) and failed to cause dengue.

Comment.—It is apparent that the immunity of these Filipinos was sufficient not only to prevent the development of symptoms of dengue, but to prevent sufficient development of the virus to infect normal mosquitoes.

Experiment 8.—August 6, 1929. Ten Philippine Scout soldiers were each bitten by from eight to ten infective *A. ægypti* of lots 30 and 32, which had transmitted dengue to American volunteers (A.V. 20–B and A.V. 28–P, respectively). Six days later, August 12, each of these Fili-

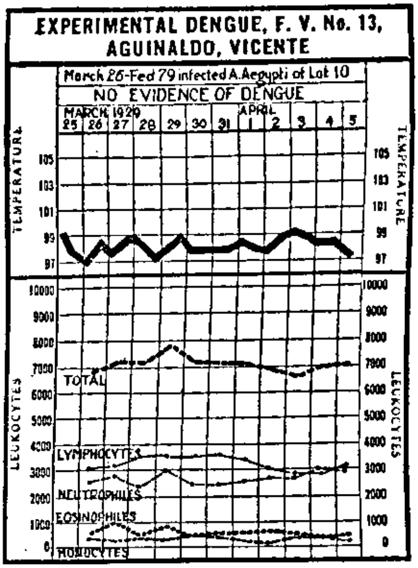

FIG. 42. Experimental dengue in Filipino volunteer 13.

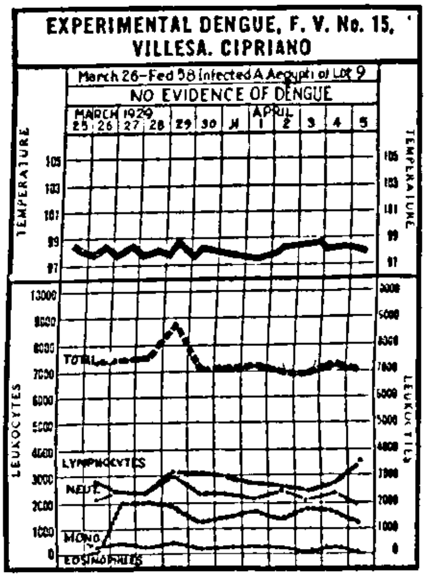

FIG. 44. Experimental dengue in Filipino volunteer 15.

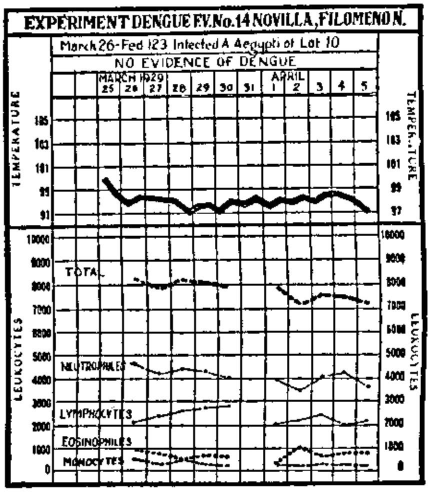

FIG. 43. Experimental dengue in Filipino volunteer 14.

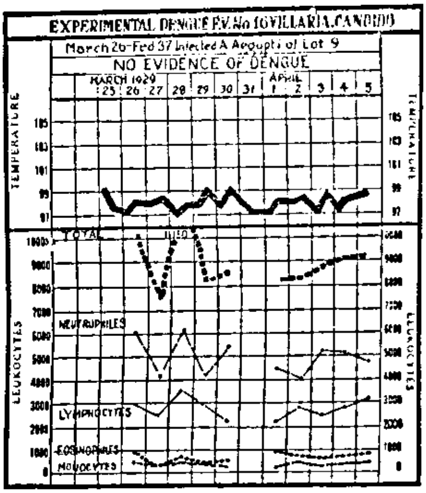

FIG. 45. Experimental dengue in Filipino volunteer 16.

pinos was inoculated intravenously with 0.5 cubic centimeter amounts of citrated infective blood collected about two hours earlier from an experimental case of dengue (A.V. 27–P), during the first day of the disease. Records of daily temperatures and total leukocyte counts were made between August 6 and 21. Several of the men had temperatures as high as 99 to 99.8° F. at irregular intervals, but none of them complained of any illness.

In two instances the leukocyte counts were less than 6,000 on the third and fourth days. There was no clinical evidence to indicate that any of these men developed dengue, either from the bites of infective mosquitoes or from the subsequent intravenous inoculations with infective dengue blood.

Nevertheless, different groups of normal *A. ægypti*, called lots 93 *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, and *i*, respectively, were allowed to feed daily on each man during the third, fourth, fifth, and sixth days after the bitings with infective mosquitoes, while other groups designed as lots 94 *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, and *i*, respectively, were fed on the ten men during the fourth, fifth, sixth, and seventh days after the blood inoculations.

The ten cages containing the various sublots of *A. ægypti* No. 93 were applied August 26 and again September 5 to the arms and legs of a susceptible American volunteer (A.V. 37–M). On the latter date forty-five, forty-three, forty-five, thirty-seven, forty-one, forty-two, thirty-eight, twenty, thirty-six, and fifty of lot 93 mosquitoes, respectively, took blood. As dengue did not result from these feedings, and the volunteer was later proven susceptible, it was considered likely that infective virus had not been present in the blood of the Filipinos, on the third, fourth, fifth, or sixth days after the biting experiments.

Eight of the ten sublots of No. 94 were tested similarly for infectivity September 5 by feeding them on a susceptible volunteer (A.V. 41–F). Of the respective sublots, twenty-eight, thirty-seven, thirty, twenty-six, forty-one, twenty-five, forty-two, and eighteen mosquitoes bit this volunteer but failed to cause dengue. The susceptibility of A.V. 41–F was later proved by the fact that he developed typical dengue following the feeding of a known infected lot of *A. ægypti.*

Comment.—It is believed that these ten Filipinos were completely immune to dengue.

From these various experimental attempts to produce dengue in adult Filipinos living in endemic areas, it may be concluded that such individuals are usually immune and that their immunity is sufficiently strong to protect them against relatively large amounts of infective virus.

(B) THE SUSCEPTIBILITY TO DENGUE OF NATIVES LIVING IN LOCATIONS WHERE THE DISEASE IS NOT ENDEMIC

In order to obtain more information as to whether immunity to dengue might be a natural racial characteristic of natives of the Philippines, transmission experiments were carried out with other natives living near Baguio in the mountains of northern Luzon, a locality that is supposed to be free from dengue. Before these experiments were begun an Igorot was seen suffering with dengue contracted in the lowlands. His history was as follows:

A case of naturally acquired dengue in a Bontoc Igorot.—April 17, 1929, a Bontoc Igorot boy, Howard Fomokao, about 22 years of age, reported to the hospital at Camp John Hay, Mountain Province, with a temperature of 100.2° F., a pronounced generalized punctate and confluent macular rash, and a typical history of dengue. A leukocyte count taken at this time indicated a definite leukopenia; total 4,550, lymphocytes 58 per cent, and granulocytes 42 per cent. Other examinations were negative. This individual had lived in Bontoc at an elevation of over 5,000 feet, until 1919, when he moved to Baguio (elevation about 5,000 feet). He stayed in Baguio until 1927 when he went to Fort William McKinley (elevation not more than 125 feet) where he worked as a "house-boy." He was never ill until Sunday, April 14, 1929, at which time he had started to return to Baguio. During the trip up the mountain he became feverish and dizzy, his eyes hurt, and all his bones and joints ached. There was no rash at that time. Monday, April 15, the aching was less severe, but his eyes burned and the calves of his legs felt stiff. Tuesday, April 16, he had no pain, but felt weak and dizzy. He was seen at Camp John Hay Wednesday (April 17) during the last day of a typical case of dengue. The following day (Thursday, April 18) his temperature was normal and he felt well; however, his leukocyte count was still low: Total cells 3,975, lymphocytes 45 per cent, granulocytes 50 per cent. Blood collected this date was sent by mail to Manila where it was inoculated into a susceptible volunteer with negative results. However, this failure does not indicate that the infection was not dengue, as the blood was collected late in the disease and was not inoculated for at least thirty-six hours after collection.

Comment.—The susceptibility of this Igorot, indicated by a typical case of dengue was, no doubt, due to the fact that he had spent all of his previous life in a dengue-free locality and had not been infected previously. Dengue was contracted at Fort William McKinley or in Manila and developed during his return to the mountains.

EXPERIMENTAL

After some difficulty four of the rather primitive Ifugao boys, from the barrio at Camp John Hay, near Baguio, agreed to volunteer for the following transmission experiments.

Experiment 9.—April 21, 1929, 5 p. m. F.V. 1- "Thomas," an Ifugao Igorot boy about 15 years old, who was born at Banaui and had never been out of the mountains, was bitten by seven infective *A. ægypti*, lot 9, which fifty-six days earlier had ingested blood from an experimental case of dengue (A.V. 3–A), during the first day of the disease. Mosquitoes of this same lot had already been proved infective by transmission experiments with an American volunteer (A.V. 6–R). After an incubation period of six days, Thomas developed typical symptoms of a severe case of dengue. The temperature reached a peak of 103.4° F. the afternoon of April 27 and after a gradual decline to normal April 30 again rose to 103.6° May 2. The total leukocyte counts, which had been high during the incubation period, showed a steady decrease after the beginning of the fever, reaching 4,700 the afternoon of April 30. Headache, and pain in the eyes and joints, were extreme. April 28, both parotid glands became so swollen as to suggest the appearance seen in mumps, but this swelling subsided within a few days. The duration of the fever was five and one-half days.

Experiment 10.—April 21, 1929, 5 p. m. F.V. 2- "Banig," an Igorot boy about 17 years old, who was born at Banaui, and had never been out of the mountains, was bitten by six infective *A. ægypti*, lot 9. At the time of this biting Banig had a respiratory infection with a temperature of 101° F. and a leukocyte count of 10,000. By April 25 both temperature and leukocyte counts were normal. After an incubation period of five and one-quarter days dengue began the afternoon of April 27, with headache, pain in eyes, bones, and joints, and a temperature of 101.2° F. From this time on there was a steady decline in the total leukocyte count, which was 4,700 the morning of April 29. In this case the fever lasted four days.

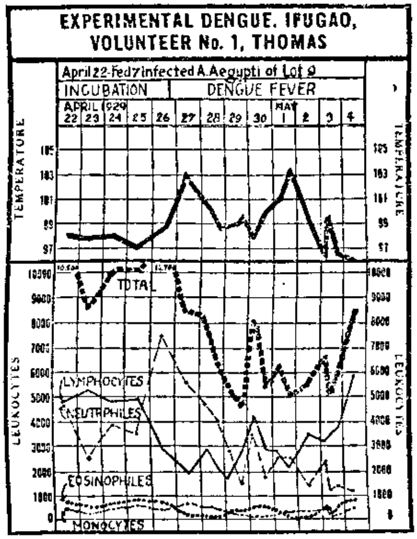

FIG. 46. Experimental dengue in Ifugao volunteer 1.

Experiment 11.—April 21, 1929, 5 p. m. F.V. 3- "Pusayen," an Ifugao Igorot boy about 17 years old, who was born at Banaui and had never left the mountains, was bitten by six infective *A. ægypti*, lot 9. The following day the ring forms of *Plasmodium falciparum* were found in blood smears. After an incubation period of five and one-quarter days dengue began and Pusayen was admitted to the hospital with headache, backache, pains in the eyes, temperature 102.4° F., and a leukocyte count of 5,900. There was some enlargement of the parotid glands. The fever persisted, and April 28 Pusayen had profuse nose bleed twice during the afternoon. The morning of May 1 he was discharged from the hospital but returned that afternoon with renewal of symptoms and was readmitted the next day. The duration of fever in this case was five days. Leukopenia lasted for five days and reached a low point of 4,600.

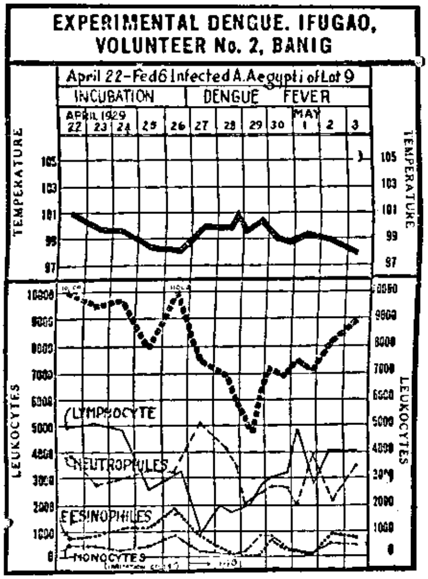

FIG. 47. Experimental dengue in Ifugao volunteer 2.

Experiment 12.—April 26, 1929. F.V. 4- "Basilan," an Ifugao Igo-

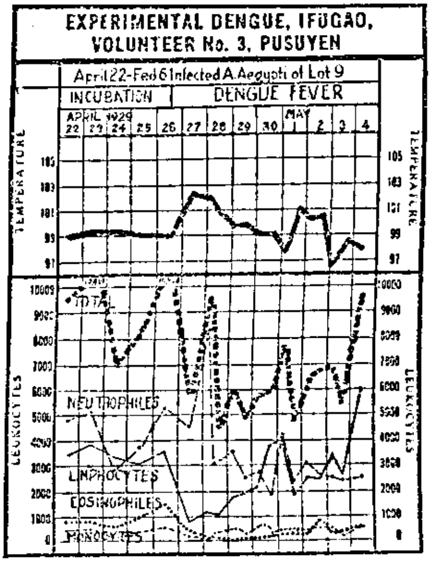

FIG. 48. Experimental dengue in Ifugao volunteer 3.

rot boy about 17 years old, who was born in Banaui and had never been out of the mountains, was bitten by five infective *A. ægypti*, lot 9. After about four days incubation dengue fever developed. The exact time cannot be determined accurately as "Basilan" failed to report April 30 and May 1, and stated that he had been sick in his house in the barrio. When admitted to hospital May 2, he had symptoms of dengue which persisted for four more days.

COMMENT

The last four experiments in which a few infective mosquitoes produced typical dengue in each of four natives from a part of the Philippines that is probably free from the disease, indicate that the immunity observed in natives living in endemic areas is probably acquired by previous infection It is believed that this explanation, advanced by Siler, Hall, and Hitchens, is probably correct and that the natives in the vicinity of Manila and elsewhere probably become infected during childhood or early adult life and consequently develop a strong immunity to dengue. It is of interest to note that lower animals, including mice, guinea pigs, rabbits, and monkeys living in places where they may be exposed to dengue have invariably been just as resistant to experimental infection as were the Philippine Scout volunteers, while certain monkeys from Japan

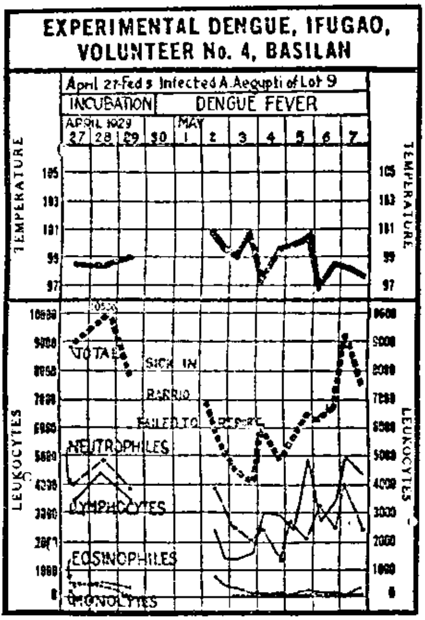

FIG. 49. Experimental dengue in Ifugao volunteer 4.

and from the mountains of northern Luzon have been proved susceptible. In one instance, it was proved that a Japanese monkey (*Macacus fuscatus* 2–W) developed immunity as a result of an infection transmitted by lot 52 *Aëdes ægypti,* about two weeks previously.

Considering the wide distribution of dengue and the many opportunities for mild or unrecognized infections in man and lower animals, it will require more-conclusive evidence than has been offered in the past to prove that immunity to dengue, even in animals other than man and monkey, is natural rather than acquired.

PART II. CLINICAL AND EPIDEMIOLOGICAL OBSERVATIONS CONCERNING THE POSSIBILITY OF REPEATED INFECTIONS WITH DENGUE

For more than one hundred years various observers have called attention to the occurrence of second, third, and fourth attacks of clinical dengue in a certain proportion of individuals. Dengue relapses and recurrences within short periods of time have been reported by Kennedy(15) (1825), Sandwith(1) (1888), Skattowo(8) (1890), Godding(2) (1890), and others. However, Ashburn and Craig(13) in 1907 made the following remarks concerning the possibility of mistakes in reporting recurrences of dengue:

> The correctness of reports of cases in which attacks have occurred a month apart we very much doubt. We had about six patients sent back to us after such periods, supposed to be suffering from second attacks, but in no case was it so. The "second attack" was usually a "malarial proxysm." Cleland(16) (1923) observed, concerning dengue, that one or two relapses may occur after a primary attack, and that second attacks after an interval of a month or longer are probably reinfections; but "neither relapses nor early reinfections are common." He also remarked that "considerable diversity of opinion exists as to the duration of immunity, some considering there is little or none, and others that a previous attack may afford some considerable degree of protection after 21 and even 32 years."

Siler, Hall, and Hitchens(12) (1926), after a careful analysis of the records of several hundred American soldiers stationed in Manila, arrived at the following conclusions:

> Our second epidemiological study indicates that under military conditions in Manila, persons coming from the United States are attacked during their first year to the extent of about 40 per cent. Of those who had one attack, 30 per cent may expect a second; of those who have had two attacks a certain percentage may expect a third; a few may have

four attacks, but our figures are not sufficiently large to make a percentage estimate more than suggestive.

In 1929 the present Research Board made use of a questionnaire sent to a selected group of the leading American and European civilian residents of Manila in an attempt to obtain data concerning their personal experiences with dengue fever. Three hundred seventy-nine individuals who had lived in the Philippines for periods of from one to thirty-five years answered the questions, which included age, birthplace, length and places of residence in the Philippines, number of attacks of dengue in the Philippines and elsewhere, including dates, clinical symptoms, and remarks as to whether or not the diagnosis was made by a physician, and if so whether leukocyte counts were made. The results of an analysis of these reports are shown in Table 22.

The fact that only 63.9 per cent of the "infections" reported were diagnosed by physicians, and that the diagnosis was confirmed by leukocyte counts in only 5.7 per cent, of course, detracts from the value and accuracy of these observations. However, the report furnished by Dr. H. E. Stafford, who has been one of the leading physicians of Manila since the Spanish-American War, is of decided interest. Doctor Stafford stated that he had had two or possibly three attacks of dengue, the first in 1900, the second about 1903, and the third before 1906, each characterized by fever of about five days duration, with aches or pains in the head, eyes, bones, and muscles, moderate aching of the joints, slight chilliness at onset, and no digestive disturbances. Rash did not occur at onset, but was present at end of fever, which was not of the "saddle back" type. The diagnosis was made from the appearance of the rash, and leukocyte counts were not made. He also added the following remark: "This was the usual history of two or three hundred cases under my care in the Civil Hospital when I was chief surgeon of the same."

From the various clinical and epidemiological reports and opinions available on the subject, it appears that there is a widespread belief that many individuals may suffer with more than one attack of dengue in a short period of time. If correct this could indicate that the immunity produced by a single attack may be slight and of short duration.

However, a careful analysis of the clinical records of patients admitted to Sternberg General Hospital between April 1, 1927,

TABLE 22.—*Reinfections with "dengue." Report of personal experiences of residents of Manila.*

Residence in Philippines.	Number of persons.	"Never had dengue."		"Had only one attack."		"Had two attacks."		"Had three attacks."		"Had four attacks."	
Years.			*P.ct.*		*P.ct.*		*P.ct.*		*P.ct.*		*P.ct.*
1	23	10	43.5	13	56.5	----	----	----	----	----	----
2	28	10	35.71	12	42.85	6	21.43	----	----	----	----
3	31	12	38.70	12	38.70	7	22.57	----	----	----	----
4	15	5	33.33	6	40	4	26.66	----	----	----	----
5	18	6	33.3	9	50	3	16.7	----	----	----	----
6	14	3	21.43	7	50	4	28.57	----	----	----	----
7	9	2	22.22	1	11.11	3	33.33	3	33.33	----	----
8	13	6	46.15	3	23.07	2	15.38	2	15.38	----	----
9	9	2	22.22	4	44.44	2	22.22	1	11.11	----	----
10	21	6	28.57	5	23.80	7	33.82	3	14.28	----	----
11	11	----	----	5	45.45	4	36.36	2	18.18	----	----
12	2	1	50	----	----	----	----	1	50	----	----
13	3	1	33.3	1	33.3	1	33.3	----	----	----	----
14	6	2	33.33	1	16.66	1	16.66	2	33.33	----	----
15	6	1	16.6	3	49.8	1	16.6	1	16.6	----	----
16	8	3	37.5	3	37.5	1	12.5	----	----	1	12.5
17	9	4	44.44	3	33.33	1	11.11	1	11.11	----	----
18	12	3	24.99	4	33.33	2	16.66	2	16.66	1	8.33
19	11	3	27.27	6	54.54	2	18.18	----	----	----	----
20	16	6	37.50	4	25	4	25	2	12.50	----	----
21	8	1	12.5	4	50	3	37.5	----	----	----	----
22	7	2	28.57	2	28.57	3	42.85	----	----	----	----
23	7	3	42.85	4	57.15	----	----	----	----	----	----
24	6	2	33.34	4	66.66	----	----	----	----	----	----
25	6	3	49.98	1	16.66	----	----	1	16.66	1	16.66
26	8	3	37.5	5	62.5	----	----	----	----	----	----
27	7	2	28.57	3	42.85	1	14.28	1	14.28	----	----
28	8	3	37.5	4	50	----	----	1	12.5	----	----
29	18	4	22.22	8	44.44	4	22.22	2	11.11	----	----
30	16	6	37.50	5	31.25	1	6.25	4	14.1	----	----
1–5	115	43	37.3	52	45.4	20	17.3	----	----	----	----
6–10	66	19	28.78	20	30.3	18	27.27	9	13.63	----	----
11–15	28	5	17.85	10	35.71	7	25	6	21.42	----	----
16–20	56	19	33.93	20	35.72	10	17.86	5	8.93	2	3.57
21–25	34	11	32.35	15	44.1	6	17.6	1	2.9	1	2.9
26–30	57	18	31.57	25	43.85	6	10.5	8	14.1	----	----
31–35	23	10	43.5	6	26.1	3	13.04	2	8.69	2	8.69
1–35	379	125	32.98	148	39.0	70	18.47	31	8.18	5	1.32

and April 1, 1930, fails to entirely substantiate this opinion. The results of this study, which was made by Capt. Mack M. Green, M. C., attending surgeon for the City of Manila, are given below.

AN ANALYSIS OF REINFECTIONS REPORTED AMONG 1258 CASES DIAGNOSED "DENGUE FEVER" AT THE STERNBERG GENERAL HOSPITAL, MANILA, P. I.

"From an analysis of the records of the Sternberg General Hospital, Manila, P. I., between April 1, 1927, and April 1, 1930, it was found that over one thousand two hundred fifty-eight individuals were admitted with a diagnosis of dengue. Eighty-three of these individuals had been readmitted to the hospital at various times and given a second diagnosis of dengue by the ward surgeon, while four came into the hospital a third time with conditions which were called dengue. A careful study of the hospital records of the eighty-three persons readmitted showed that only twenty of them had leukocyte counts below 6,000 during both periods of hospitalization. As a rule only one blood count, taken on the day of admission to the ward, was recorded.

"The following table (No. 23) shows the results of this study.

"As shown above, patients 3 and 20 became ill with symptoms of dengue one week after their discharge from hospital, and it is believed that they may be considered as relapses. Patient 6 had a mild headache, temperature 101, and cramplike pains in abdomen on admission, with no other symptoms. The fever lasted only twenty-four hours and was normal until date of discharge four days later. It is noted from the clinical record that this patient's symptoms began after a midnight meal of sandwiches and beer and it seems quite probable that the diagnosis should have been gastritis, instead of dengue. The diagnosis was presumably made because of the headache and leukopenia. Patient 8 was diagnosed as dengue on three different occasions. The last two admissions, which were separated by an interval of only nine days, probably represented a relapse. Patient 14, on second admission, had a mild headache with no other symptoms. This man remained in the hospital twenty-two days and from a study of the clinical record apparently had a mild back strain. The diagnosis of dengue was made from symptoms of backache and from the white blood count. Patient 15 was a syphilitic case of long standing who had been given four complete courses of treatment with salvarsan and mercury. This patient was admitted several months later to another ward with cerebro-spinal syphilis.

"From a study of these clinical records and from the above findings, I believe it is unwise in a great many cases to make a diagnosis of dengue from a clinical standpoint alone or from a single white blood count.

"Dengue must be differentiated from measles, influenza, cerebro-spinal meningitis, cerebro-spinal syphilis, typhus and typhoid fever, acute rheumatic fever, arthritis, seven-day fever, syphilitic roseola, phlebotomus fever, malaria, and yellow fever.

CONCLUSIONS

1. "This study, based on the hospital records of 1,258 individuals diagnosed as 'dengue' during a period of three years, shows that only 6.5 por cent were subsequently ill with conditions called 'dengue,' and of those readmissions only 1.5 per cent had leukocyte counts below 6,000 during both the first and later illnesses.

2. "A critical review of the records of the twenty patients who had leukopenia during both the first and subsequent admissions to hospital indicate that even in certain of these there was some doubt as to whether dengue had occurred more than once. In two of the individuals the intervals between attacks were so short that they might have represented relapses of the original infection; nine patients had other infections, including coryza, pharyngitis, tonsillitis, pleurisy, gastro-enteritis, and syphilis, which could have caused confusion in the diagnosis, while the remaining nine patients had clinical symptoms of dengue both times. Thus only nine, or 0.072 per cent, of the 1,258 individuals were readmitted for what appeared clinically to be typical reinfections with dengue."

This contribution by Captain Green emphasizes the difficulties often encountered, even under the best of conditions, in arriving at an accurate clinical diagnosis. Because of the added possibility that certain febrile infections showing all of the symptoms, including the rash and the progressive leukopenia considered typical of dengue, may be due to entirely different eitological agents or to denguelike viruses, it is obvious that any final conclusions concerning the specific immunity produced by a single attack of dengue should be based on controlled experimental attempts to reinfect individuals who have previously had typical dengue, caused experimentally by a known strain of dengue virus.

Several workers have attempted to produce experimental dengue in individuals who, according to their histories, had al-

TABLE 23.—*A clinical analysis of twenty cases reported as dengue reinfections. Based on the records of 1,258 individuals with diagnosis of dengue from Sternberg General Hospital, April 1, 1927, to April 1, 1930.*

[Eighty-three, or 6.59 per cent, of the patients were readmitted with the same diagnosis and only twenty of these cases, or 1.58 per cent, had leukocyte counts lower than 6,000.]

Patient No.	First illness.						Interval between first and second days of illness.	Second illness.						Remarks.
	Duration.		Temperature.		Leukocytes; lowest count.	Complications or additional diagnosis.		Duration.		Temperature.		Leukocytes, lowest count.	Complications or additional diagnosis.	
	Before admission to hospital.	After admission to hospital.	Maximum.	Duration.				Before admission to hospital.	After admission to hospital.	Maximum.	Duration.			
	Days.	*Days.*	°F.	*Days.*				*Days.*	*Days.*	°F.	*Days.*			
1	5	6	100.4	3	4,700	None	91	6	8	100.2	4	2,550	None	Both admissions were clinically dengue.
2	1	8	101.4	5	4,650	do	210	3	7	101.0	1	5,300	Coryza	Had mild coryza and pharyngitis on second admission.
3	1	4	102.0	2	3,500	do	7	4	6	102.0	3	3,050	None	Second admission is believed to be a relapse of first.
4	1	6	100.2	2	4,450	do	370	1	10	105.0	7	4,950	Pleurisy, acute, left base.	Leukocyte count on admission of second attack 4,950, three days later was 8,050.
5	3	7	102.4	4	3,850	do	30	6	8	103.6	3	4,650	None	Immunity of short duration if both admissions were dengue.
6	0	5	101.0	1	5,250	do	227	1	5	101.6	4	3,800	do	Only symptom on first attack was mild headache and pain in abdomen.
7	1	7	100.4	3	4,150	do	343	3	6	103.6	4	5,200	do	Both admissions were clinically dengue.
8	1	6	102.0	2	4,750	do	165	?	8	104.0	3	5,700	do	Discussed in the text.

9	1	8	102.0	4	2,100	do	369	3	4	100.6	2	5,250	Rhinitis, acute	The diagnosis of influenza cannot be ruled out on second admission.
10	1	10	102.4	6	4,250	do	27	?	5	101.6	2	5,150	None	Immunity of short duration if both admissions were dengue.
11	2	12	101.2	8	4,600	Tonsilitis, acute	366	2	7	102.4	5	4,450	do	Had mild tonsilitis on first admission.
12	1	9	104.0	7	3,600	None	334	3	3	101.4	3	2,800	do	Had temperature of 99°F. when discharged.
13	2	13	103.0	7	3,800	do	77	2	9	102.4	6	4,250	do	Both admissions were clinically dengue.
14	1	4	102.0	2	4,600	do	433	3	22	101.0	16	4,200	Pharyngitis and sacroilliac strain	Diagnosis of dengue on second admission made from leukocyte count and backache, mild.
15	2	7	102.2	5	5,150	do	30	2	11	100.4	8	5,550	Tonsilitis, acute	Syphilis.
16	0	9	103.8	6	3,400	do	348	1	5	101.2	4	4,100	None	
17	?	7	102.4	3	5,200	do	256	0	5	100.2	2	5,200	do	Mild form of dengue on second admission.
18	1	8	103.6	3	2,800	do	207	2	5	101.4	2	5,800	do	Both admissions were clinically dengue.
19	3	4	102.8	3	3,900	Tonsilitis mild	76	4	6	102.0	4	4,350	Bronchitis, mild	
20	1	4	103.6	3	5,500	None	6	2	6	103.0	2	4,350	None	Second admission is believed to be a relapse of first.

ready suffered with naturally acquired infections. For example, Ashburn and Craig(13) (1907) induced dengue in one individual who said he had had three attacks, the last, two and one-half years previously. Siler, Hall, and Hitchens(12) (1926) inoculated dengue virus into each of eight individuals, who, according to their clinical records, had suffered with spontaneous attacks diagnosed as dengue fifty to one hundred forty-eight days earlier. As a result of the experimental inoculations, four, or 50 per cent, of these men developed dengue.

PART III. EXPERIMENTAL OBSERVATIONS CONCERNING THE IMMUNITY PRODUCED BY A SINGLE ATTACK OF DENGUE

In striking contrast to the general opinion concerning the shortness of dengue immunity, based on clinical and statistical observations, are the results of the following studies in which individuals who had suffered from experimentally induced attacks of dengue, were later tested for immunity by the inoculation of materials known to contain infective dengue virus. Cleland, Bradley, and McDonald(13) (1918), using infective serum and corpuscles from a case of dengue, inoculated 0.5 cubic centimeter subcutaneously into a volunteer, who twenty-four days previously had had a typical attack, produced by a similar injection of infective materials. This man was found to be immune. Later (1919), these authors(3) inoculated about 0.5 cubic centimeter of diluted infective serum in an attempt to reinfect an individual in whom dengue had been produced experimentally by mosquitoes seven and one-half months previously. Negative results were also obtained in this experiment, indicating that the immunity produced by the experimental attack protected against reinfection for at least 229 days.

Siler, Hall, and Hitchens(12) (1926) inoculated 0.5 cubic centimeter amounts of infective dengue virus into each of eleven volunteers, all of whom had been infected experimentally with dengue from thirty-two to sixty-eight days previously. Of this group one man whose initial dengue had occurred fifty-three days earlier developed a mild infection lasting two days; while the other ten, whose experimental infections had taken place thirty-two, forty, forty-six, forty-eight, fifty-three, sixty, sixty, sixty-seven, and sixty-eight days earlier, were all immune. Manoussakis(17) (1928), after failing to reinfect nine experimental volunteers, concluded that persons who had recently recovered from dengue fever were resistant to doses of infective serum after two months. Blanc and Caminopetros(11) (1929) also considered dengue immunity from an experimental standpoint. They reported that ten months after the experimental production of dengue in twenty-five volunteers, nine of these individuals were reinoculated with large doses of dengue virus. None of them showed any signs of infection following the second inoculation, and it was concluded that dengue probably confers a high degree of immunity which is of considerable duration.

PRESENT INVESTIGATION

During the various studies of dengue conducted by this board since December, 1928, a total of more than eighty-three American volunteers have been infected experimentally with a single strain of virus. Although these men continued to live in a locality where dengue is endemic, only one of them developed what might have been a second infection during the subsequent periods of from one to fifteen months. Several of the volunteers returned to hospital with fever and were suspected as reinfections at the time of admission, but subsequent observation and examination proved that they were suffering from other acute infections rather than dengue. The diagnosis in the one doubtful case was not confirmed. As indicated in Table 24, experimental attempts were made to reinfect thirty-five of the volunteers, all of whom had been infected previously with the same strain of virus transmitted by mosquitoes. While the numbers of mosquitoes used in producing the infections differed in the various experiments, ten or less were used in most of the immunity tests. The intervals between the attacks of dengue and the tests for immunity varied from twelve to four hundred days. One of these men was readmitted to hospital two days after the test with fever due to another cause. Among the thirty-five persons rebitten by infective mosquitoes not a recognizable dengue reinfection occurred.

Comment.—The results obtained in these experiments show that for periods of from one-half to thirteen months after an experimentally induced attack of dengue, 100 per cent of the thirty-five volunteers were immune to reinfection. Therefore, it is believed that the immunity produced by a typical attack of dengue is usually strong and of considerable duration.

TABLE 24.—*Immunity tests following experimental dengue fever.*

Experiment No.	Volunteer.	Volunteers tested for immunity.									Results.
		Infected by—				Days elapsing	Retest with—				
		Number fed.	Species of mosquitoes.	Lot No.	Date.		Number fed.	Species of mosquitoes.	Lot No.	Date.	
1	A.V. 3-A	56	*Aëdes ægypti*	3	Feb. 19, 1929	70	8	*Aëdes ægypti*	9-10	May 1, 1929	Immune.
2	A.V. 4-Y	56	do	2	Feb. 19, 1929	86	8	do	12	May 16, 1929	Do.
3	A.V. 24-H	10	*Aëdes albopictus*	29	Aug. 7, 1929	180	8	do	135	Feb. 3, 1930	Do.
4	A.V. 38-H	203	*Aëdes ægypti*	92	Sept. 5, 1929	151	8	do	135	Feb. 3, 1930	Do.
5	A.V. 39-M	47	do	44-47	Sept. 5, 1929	151	10	do	135	Feb. 3, 1930	Do.
6	A.V. 13-W	85	do	18	May 17, 1929	262	10	do	135	Feb. 3, 1930	Do.
7	A.V. 30-L	16	do	16	Aug. 17, 1929	171	19	do	135	Feb. 4, 1930	Do.
8	A.V. 33-B	33	*Aëdes albopictus*	49	Sept. 14, 1929	143	14	do	135	Feb. 4, 1930	Do.
9	A.V. 29-J	(b)	*Aëdes ægypti*	32	Aug. 17, 1929	171	8	do	135	Feb. 4, 1930	Do.
10	A.V. 2-M	15	do	1	Dec. 31, 1928	400	9	do	135	Feb. 4, 1930	Do.
11	A.V. 4-Y	56	do	2	Feb. 19, 1929	351	8	do	137	Feb. 5, 1930	Do.
12	A.V. 3-A	56	do	3	Feb. 19, 1929	351	15	do	137	Feb. 5, 1930	Do.
13	A.V. 36-V	28	do	3	Sept. 21, 1929	137	9	do	137	Feb. 5, 1930	Do.
14	A.V. 32-W	22	*Aëdes albopictus*	43	Aug. 26, 1929	171	14	do	137	Feb. 11, 1930	Do.
15	A.V. 34-S	2	do	43	Aug. 31, 1929	164	9	do	137	Feb. 11, 1930	Do.
16	A.V. 14-A	12	*Aëdes ægypti*	19	June 20, 1929	268	14	do	161	Mar. 15, 1930	Lot not proved infected.
17	A.V. 20-B	9	do	30	July 29, 1929	229	11	do	166	Mar. 15, 1930	Do.
18	A.V. 25-D	3	*Aëdes albopictus*	29	Aug. 6, 1929	220	10	do	167	Mar. 14, 1930	Immune.
19	A.V. 26-T	8	do	29	July 29, 1929	245	15	do	174	Mar. 31, 1930	Do.
20	A.V. 55-S	8	*Aëdes ægypti*	71	Oct. 26, 1929	172	7	do	190	Apr. 16, 1930	Do.
21	A.V. 76-M	61	do	137	Feb. 1, 1930	76	6	do	190	Apr. 16, 1930	Do.
22	A.V. 56-K	25	do	104	Nov. 16, 1929	151	9	do	190	Apr. 16, 1930	Do.
23	A.V. 54-K	54	do	97	Oct. 31, 1929	167	10	do	190	Apr. 16, 1930	Do.

24	A.V. 6-R	49	do	9	Apr. 11, 1929	369	10	do	190	Apr. 16, 1930	Do.
25	A.V. 75-P	60	do	138	Feb. 3, 1930	71	7	do	190	Apr. 16, 1930	Do.
26	A.V. 46-L	10	do	52	Oct. 2, 1929	196	9	do	190	Apr. 16, 1930	Do.
27	A.V. 57-E	30	do	96	Nov. 11, 1929	156	9	do	195	Apr. 17, 1930	Do.
28	A.V. 37-M	42	do	53	Sept. 14, 1929	215	5	do	195	Apr. 17, 1930	Do.
29	A.V. 77-E	87	do	162	Feb. 17, 1930	65	11	do	195	Apr. 23, 1930	Do.
30	A.V. 50-C	14 and 1	do	75 and 80	Oct. 21, 1929	186	15	do	195	Apr. 25, 1930	Do.
31	A.V. 61-J	84	do	116	Dec. 7, 1929	139	7	do	195	Apr. 25, 1930	Do.
32	A.V. 66-W	109	do	114	Dec. 10, 1929	136	5	do	195	Apr. 25, 1930	Do.
33	A.V. 53-C	6 15 4 6 15 9	do	82 83 84 85 88 89	Oct. 28, 1929	179	5	do	195	Apr. 25, 1930	Do.
34	A.V. 48-H	64	do	71	Oct. 21, 1929	189	12	do	195	Apr. 28, 1930	Do.
35	A.V. 41-F	6	do	52	Oct. 7, 1929	203	8	do	195	Apr. 28, 1930	Do.
36	A.V. 47-D	50	do	69	Oct. 6, 1929	12	11	do	52	Oct. 19, 1929	Do.
37	A.V. 72-E	11 56 65	do	125 126 145	Jan. 27, 1930	12	8	*Aëdes albopictus*	128	Feb. 7, 1930	Do.
38	*Macacus fuscatus* 2-W.	44	do	52	Oct. 7, 1929	(a)					

[a] Twenty-six days later, November 1, 5 cubic centimeters of dengue blood from A.V. 51-S were injected.

[b] Filtrates of 50.

DISCUSSION

No evidence has yet been advanced to prove that all people of any race or group may be naturally immune to dengue. This statement probably applies also to many of the published observations concerning the immunity of lower animals. Following the introduction of dengue into a locality, a large proportion of the inhabitants become infected; and in places where dengue has existed for some time a large proportion of the adult natives and the lower animals are found to be resistant to the infection. This is the case in the Philippines where adult natives and lower animals living in endemic areas have been shown to be immune to dengue, while both natives and monkeys from dengue-free areas in the mountains a few hundred miles away are susceptible.

There is no information available to indicate whether the immunity of the adults living in endemic areas is due to a single previous attack of dengue during early life, or whether it has been built up as the result of a series of infections, and maintained by repeated subsequent exposures. It should be interesting to test the immunity of residents of dengue-free areas at intervals, after experimental infection. From clinical and epidemiological studies of susceptible individuals after their arrival in endemic areas, many observers have concluded that an attack of dengue often confers only slight immunity of short duration, and that a certain proportion of such individuals may have second, third, or even fourth attacks within a few years. However, the analysis of clinical records of 1,285 cases, contributed by Captain Green, indicates that second and third clinical attacks during a three-year period may be relatively infrequent.

Certain observers have succeeded in infecting individuals who according to their histories or official records had had previous attacks of dengue. However, from the experimental results reported by Cleland, Bradley, and McDonald,(3) Siler, Hall, and Hitchens,(12) Manoussakis,(17) Blanc and Caminopetros,(11) and the observations of the authors, it is believed that a single attack of dengue in a person living in an endemic locality, confers protective immunity of considerable strength and duration.

SUMMARY AND CONCLUSIONS

1. A large proportion of human beings are apparently naturally susceptible to dengue. This is indicated by the fact that in different epidemics from 75 to 100 per cent of the in-

habitants of various localities have been attacked, particularly in places where the disease is first introduced.

2. American soldiers from various parts of the United States where dengue is not endemic are known to be extremely susceptible to natural dengue infections and to experimental inoculations with the virus, while Americans who have previously lived in endemic localities are usually immune.

3. It has been observed in various parts of the world that most adult natives who have passed through an epidemic of dengue or who live in endemic areas are apparently immune. That this is true of Filipinos living in endemic areas is indicated by the low prevalence of dengue among Philippine Scout soldiers, and by the transmission experiments performed by Siler, Hall, and Hitchens and by the authors, in which between 93 and 100 per cent of over sixty volunteers were immune to inoculations of infective dengue virus.

4. In two instances the immunity of Philippine Scouts was apparently overcome by inoculations with 0.5 cubic centimeter of infective blood intravenously or with 5 cubic centimeters subcutaneously; but such was not the case in other experiments in which large numbers of infective mosquitoes were used.

5. In thirteen experiments it was shown that dengue virus introduced into immune Filipinos not only failed to cause symptoms, but failed to develop in these men, or at most to develop sufficiently to infect normal mosquitoes fed on them during ten subsequent days. This further indicates that the immunity of the natives was well developed.

6. Natives from dengue-free localities in the mountains of northern Luzon were found to be susceptible to natural and experimental dengue infection.

7. Filipinos and monkeys from Manila and other endemic areas of dengue were generally found to be immune, while those living in high altitudes in dengue-free localities (mountains of northern Luzon) were extremely susceptible.

8. Clinical and epidemiological studies have been reported, suggesting that Americans may develop two, three, four, or five attacks of dengue within a short period of time; and that the immunity produced by a single attack may be slight.

9. However, from an analysis of 1,258 hospital records of cases which occurred in Manila between 1927 and 1930, it has been indicated that the proportion of reported recurrences in which the clinical evidence was sufficient to rule out other infections was negligible.

10. Experiments in which thirty-five American volunteers, who had recovered from one attack of dengue produced by a known strain of virus, were reinoculated after periods of one-half to thirteen months gave negative results, indicate that the immunity produced by an attack of dengue probably is stronger and of longer duration than has been generally believed.

11. From these experiments it is, of course, impossible to be sure that the immunity produced by a single attack of dengue may not be maintained by subsequent introduction of virus from infective mosquitoes. This point could be settled by experimental attempts to reinfect individuals living in localities free from dengue.

12. From the results of the present epidemiological, clinical, and experimental studies, it is believed that the immunity following an attack of dengue fever in persons residing in endemic areas, is probably completely protective in a large majority of cases.

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TESTS FOR SPECIFIC ANTIBODIES IN DENGUE

In the following experiments efforts were made to demonstrate specific antibodies in serum obtained from dengue patients and from individuals known to be immune to the disease.

INJECTION OF DENGUE PATIENTS WITH SERUM FROM CONVALESCENTS

Manoussakis(1) (1928) stated that the serum of dengue convalescents failed to confer immunity to susceptible volunteers whether injected before, simultaneously with, or after the infecting dose of dengue virus. Blanc and Caminopetros(2) (1929) also reported that neither the serum nor the whole blood of convalescents had any action on the virus either in vitro or in vivo and that mixtures of such serum and dengue virus were always infective.

Experiment 1.—Two individuals during the first twenty-four hours of typical experimentally produced dengue fever were injected simultaneously with 1 and 2 cubic centimeter amounts of clear serum collected from volunteers one month after their recovery from experimental dengue. The infections continued in apparently unmodified form, and it was concluded that the serum injected had no effect on the course of the disease.

Experiment 2.—In order to determine whether the rash of dengue might be caused to disappear by intradermal injection of serum from convalescents, the following experiment was performed February 21, 1930. Blood was drawn this date, from a recently recovered case of experimental dengue whose temperature had been normal for seven days. The serum was immediately separated and 0.25 cubic centimeter was injected intracutaneously into an area of skin covered with a pronounced secondary, maculopapular rash on a volunteer (A.V. 78–O) during the fourth day of dengue fever. The wheal produced by the injection was pale momentarily, but within a few minutes again became as red as the surrounding skin. There was no fading of the rash during the following twenty hours.

PRECIPITIN TESTS

Experiment 3.—The antigens used in precipitin tests included fresh infective serum collected from experimental cases of dengue during the first day of the disease; and a mosquito filtrate obtained by grinding forty infective *Aëdes ægypti* (lot 25) with sand and 6 cubic centimeters of physiologic salt solution, filtering the suspension through cotton, and clearing the filtrate by centrifugalization. These antigens were used in 0.025 cubic centimeter amounts in small test tubes with different dilutions of serum from immune individuals who had previously been infected experimentally with dengue. No precipitation occurred either in one or eighteen hours.

COMPLEMENT-FIXATION TESTS

Tests were made for complement-fixing bodies in serum from dengue patients and from individuals immune to dengue, using several different antigens prepared with infective blood obtained from dengue patients or from mosquitoes infected with dengue virus.

Experiment 4.—An antigen was prepared with forty infected *A. ægypti* of lot 40 which had fed on an experimental case of dengue (A.V. 20–B) during the first day of the disease. The mosquitoes were macerated and suspended in 8 cubic centimeters of sterile physiologic salt solution, and after the suspension had been filtered through cotton, the filtrate was heated at 56° C. for ten minutes. This antigen, tested in various dilutions, failed to cause any fixation of complement when titrated with serum obtained from individuals who had recently recovered from experimental dengue fever.

Experiment 4–a.—Antigen 2 was prepared with 20 cubic centimeters of infective blood drawn from an experimental case (A.V. 78–O) during the first day of dengue. The blood was laked in distilled water, and centrifugalized at 2,500 revolutions per minute for forty-five minutes. The residue was diluted with a similar amount of mixture composed of equal parts of glycerin and salt solution. When diluted with equal parts of salt solution and used in complement-fixation tests with serum collected from a volunteer on the fourth day of convalescence after dengue fever, this antigen gave anticomplementary reactions. However, when a 1-to-3 dilution was used in 0.5 to 0.3 cubic centimeter amounts with known immune sera the results were negative.

Experiment 4–b.—Antigen 3 was prepared with infective blood drawn from the patient mentioned above (A.V. 78–O). Ten cubic centimeters of the coagulated blood was placed in a mortar, ground with 12.5 cubic centimeters of 95 per cent alcohol and subsequently allowed to stand for seven days. The alcoholic extract, diluted 1 to 5 with salt solution, was used in tests with serum drawn from volunteers (A.V. 24–H, A.V. 25–D, A.V. 27–P, and A.V. 28–P), and from each of the three authors, at relatively short periods after they had recovered from attacks of dengue fever. The results were negative.

Experiment 4–c.—Antigen 4 consisted of recently collected infective serum from an experimental case of dengue. This was tested with serum from individuals who had recently recovered from dengue, with negative results.

Experiment 4–d.—Antigen 5 was prepared with infective *Aëdes ægypti* of lots 9, 12, 17, 18, and 19. Two hundred seventy-nine of these mosquitoes were macerated and suspended in 5 cubic centimeters of 95 per cent alcohol. Twelve days later the alcoholic mosquito extract was cleared by centrifugalization at low speed, after which it was diluted 1 to 10 with physiologic salt solution and used in tests with sera obtained from four volunteers. Three of these individuals had recently recovered from dengue as follows: A.V. 13–W, nine days, and A.V. 3–A and A.V. 4–Y about seventy-nine days previously; while the other volunteer (A.V. 9–D) had been suffering with dengue fever for only one day. Using 0.1 cubic centimeter amounts of the 1 to 10 dilution of antigen and 0.1 cubic centimeter amounts of the four sera the tests resulted as follows: A.V. 4–Y, serum was negative; A.V. 13–W, two plus; A.V. 9–D, three plus; and A.V. 3–A, four plus.

As the remainder of this antigen was lost, another one was prepared similarly with 584 infective *A. ægypti* from various experimental lots. However, all tests with this second mosquito antigen were negative.

Experiment 4–e.—Antigens 6 and 7 were prepared from a mixture of liver and spleen taken from Philippine monkeys (*Macacus philippinensis* 33–D and 34–E) six days after the animals had been bitten by infective *A. ægypti*. The liver-and-spleen mixtures were ground up and suspended in 9 per cent salt solution in the proportion of 1 gram to 0.5 cubic centimeter. After standing overnight in the ice box the suspensions were diluted with nine times their volume of distilled water. These antigens were used in tests with sera from volunteers who had recently recovered from dengue fever. The tests were negative.

CONCLUSIONS

With the possible exception of complement-fixing bodies as noted in experiment 4–d, specific antibodies were not demonstrated in serum collected from various individuals, during and following recovery from dengue fever.

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OBSERVATIONS CONCERNING PROPHYLACTIC VACCINATION AGAINST DENGUE

From investigations in which 100 per cent of thirty-five individuals, experimentally infected with dengue, were later found to be resistant to second inoculations with infective virus, it appears that a single attack of dengue usually produces a strong immunity which may last for thirty to four hundred days and probably longer. This observation suggests the possibility of producing an effective prophylactic vaccine which might be used for the protection of susceptible individuals entering endemic localities, particularly for troops being sent for short tours of service in the Philippine Islands or other tropical countries.

Observations have been made concerning the immunizing action of subcutaneous inoculations with various materials, including noninfectious filtrates of suspensions of infective mosquitoes, dried blood collected from dengue patients during the infective period, and a vaccine prepared from a saline suspension of dengue-infected *Aëdes ægypti.*

EXPERIMENTAL

SUBCUTANEOUS INJECTIONS OF NONINFECTIOUS FILTRATES OBTAINED FROM SUSPENSIONS OF MOSQUITOES INFECTED WITH DENGUE VIRUS

During a series of experiments dealing with the filterability of dengue virus as it exists in infected mosquitoes, the virus failed to pass through diatomaceous filters in twelve instances, as was indicated by the fact that inoculation of the filtrates into susceptible volunteers did not cause dengue. When tested for immunity with infective mosquitoes, these same volunteers, with one exception, developed dengue. However, as the immunity test was applied in the excepted case several months after discharge from hospital, his record is not considered.

SUBCUTANEOUS INJECTIONS OF DESICCATED BLOOD

Several attempts were made to preserve the virus of dengue in frozen, dried blood. The virus was collected from experimental cases during the infectious stage of the disease. The dried blood, sealed in a vacuum in pyrex glass tubes, was stored at 18° C. and, after different intervals of time, five of the specimens were suspended in salt solution and tested for infectivity by subcutaneous inoculation into susceptible volunteers. As none developed dengue from this procedure, it may be assumed that the virus had either died or become attenuated. However, it seemed desirable to ascertain if such inoculations had produced any immunity. When the volunteers were tested with infective mosquitoes eight to eighteen days later, all developed dengue.

VACCINE PREPARED WITH MOSQUITOES INFECTED WITH DENGUE VIRUS

A "mosquito" vaccine (No. 1) was prepared according to the following technic and was tested on susceptible human volunteers. December 20, 1929, two thousand ten infective *Aëdes ægypti* from combined lots 95, 101, 103, 104, 110, 111, 112, 114, 116, 117, 118, 129, 130, 131, and 134, were killed and ground in a sterile porcelain mortar with fine quartz sand and 10 cubic centimeters of salt solution containing 1.6 per cent chemically pure phenol and 0.4 per cent chemically pure formalin. The suspension was transferred to a sterile bottle, and after it had stood for forty-eight hours to allow insoluble particles to settle, 10 cubic centimeters of sterile physiologic salt solution was added to the clear supernatant fluid to further dilute the preservatives. The suspension was then kept at room temperature for eight days to destroy bacteria, after which, 20 cubic centimeters of sterile salt solution was added. After slow centrifugalization, to eliminate the remaining insoluble matter, 37 cubic centimeters of a clear amber-colored fluid was obtained. This fluid, which contained phenol and formalin in final dilutions of 0.4 and 0.1 per cent, respectively, mixed with the extract from about fifty mosquitoes per cubic centimeter, was placed in ampules in 1 cubic centimeter amounts and used as a vaccine. Before use, the vaccine was examined for living bacteria and for bacterial toxins. Anaërobic and aërobic cultures were made and incubated for periods of ten or more days. Although no bacteria were found, portions of the fluid media were inoculated into guinea pigs. The vaccine was also inoculated in 1 cubic centimeter amounts directly into rabbits, guinea pigs, and white mice, all of which remained normal. As a final preliminary test, doses of the vaccine were inoculated subcutaneously into each of the authors before it was used on volunteers.

TABLE 25.—*Effect on dengue immunity of subcutaneous injections of filtrates of suspensions prepared with infected Aëdes ægypti.*

No.	Volunteer.	Inoculations with mosquito filtrates.	Test for immunity with infective mosquitoes.				Date of onset of disease.	Incubation period.	Fever.		Leukopenia.	
			Date.	Result.	Mosquitoes.				Duration.	Height.	Duration.	Lowest count.
					Number.	Species and lot No.						
								Days.	*Days.*	°*F.*	*Days.*	
1	A.V. 20-B	June 29, 1929 July 11, 1929 July 20, 1929	July 29, 1929	Dengue	9	*Aëdes ægypti*, lot 30	Aug. 4, 1929	6.00	3.5	100.4	3.5	4,200
2	A.V. 23-B	July 1, 1929 July 11, 1929 July 20, 1929	July 29, 1929	do	12	*Aëdes albopictus*, lot 29	Aug. 5, 1929	7.00	4.5	101.4	6.0	2,150
3	A.V. 24-H	July 11, 1929 July 21, 1929 (dried dengue blood).	Aug. 7, 1929	do	10	do	Aug. 12, 1929	5.50	3.5	102.0	4.0	5,100
4	A.V. 25-D	July 11, 1929 July 21, 1929 (5-day virus).	Aug. 6, 1929	do	3	do	Aug. 12, 1929	6.25	5.5	103.0	7.0	3,200
5	A.V. 27-P	July 21, 1929 (5-day virus). July 29, 1929	Aug. 6, 1929	do	1	do	Aug. 11, 1929	5.00	6.0	103.2	6.5	2,100
6	A.V. 30-L	Aug. 17, 1929	Aug. 26, 1929	do	16	*Aëdes ægypti*, lot 41	Aug. 30, 1929	4.50	4.0	103.0	7.0	3,250
7	A.V. 64-C	Nov. 29, 1929	Dec. 10, 1929	do	58	*Aëdes ægypti*, lot 117	Dec. 16, 1929	6.00	4.5	102.2	6.0	2,600

TABLE 26.—*Effect of dengue immunity of subcutaneous injections of frozen dried infective blood kept for different periods of time.*

No.	Volunteer.	Inoculation with dried dengue blood.					Volunteer tested for immunity with infective mosquitoes.		
		Date.	Amount of whole blood.	Suspended in—	Filtered.	Age of blood.	Date.	Number.	Species and lot No.
			cc.						
1	A.V. 24-H	July 21, 1929	0.5	Salt solution	Yes	4	Aug. 7, 1929	10	*Aëdes albopictus*, lot 29.
2	A.V. 26-T	July 21, 1929	0.5	do	No	4	July 29, 1929	8	Do.
3	A.V. 28-P	July 11, 1929	1.5	do	Yes	15	July 29, 1929	8	*Aëdes ægypti*, lot 32.
4	A.V. 32-W	Aug. 17, 1929	1.0	do	No	52	Aug. 26, 1929	22	*Aëdes albopictus*, lot 43.
5	A.V. 65-F	Nov. 29, 1929	0.7	do	No	79	Dec. 10, 1929	10	*Aëdes ægypti*, lot 112.

No.	Volunteer.	Volunteer tested for immunity with infective mosquitoes.			Fever.		Leukopenia.		Rash.
		Result.	Date of onset of the disease.	Incubation period.	Duration.	Height.	Duration.	Lowest count.	
				Days.	*Days.*	°F.	*Days.*		
1	A.V. 24-H	Dengue	Aug. 12, 1929	5.5	3.5	102.0	4	5,100	Terminal.
2	A.V. 26-T	do	Aug. 4, 1929	6.5	1.5	101.0	4	3,900	Initial.
3	A.V. 28-P	do	Aug. 4, 1929	6.0	4.5	102.6	5	3,700	None.
4	A.V. 32-W	do	Aug. 31, 1929	5.0	5.0	104.6	7	2,150	Do.
5	A.V. 65-F	do	Dec. 17, 1929	6.0	5.5	102.4	6	3,150	Initial and terminal.

Experiment 1.—February 13, 1930. One cubic centimeter of dengue vaccine 1 was injected subcutaneously into the right arm of a susceptible volunteer (A.V. 84–C). The only reaction noted was temporary itching at the point of inoculation. February 17, 1930, another dose of 1 cubic centimeter of vaccine was injected subcutaneously. February 24, 1930, the volunteer was tested for immunity, by allowing twenty-one infective *A. ægypti* of lot 137 to take blood at 10.30 a. m. After an incubation period of five days dengue developed March 1, 1930. The duration of the fever was five days, and the highest temperature was 102.4°, while the leukopenia lasted six days and the lowest count was 3,600. Comment: Two subcutaneous injections of vaccine 1, given eleven and seven days before the feeding of infective mosquitoes, failed to prevent the development of dengue in this volunteer.

Experiment 2.—February 24, 1930, 10.30 a. m. One cubic centimeter of dengue vaccine 1 was inoculated subcutaneously into the arm of a susceptible volunteer (A.V. 83–R). Intense itching was felt about the point of injection for a few minutes, but no other reaction was noted. March 1, 1930, a second subcutaneous injection of 1 cubic centimeter of dengue vaccine 1 was given. March 8, 1930, or twelve days after the initial injection of vaccine, the volunteer was tested for immunity by feeding eleven infective *A. ægypti* of lot 168 at 10 a. m. After an icubation period of five days, dengue fever began March 13. The fever lasted four and one-half days and the highest point reached was 102.6°, while the leukopenia lasted for five days and the lowest count was 2,600. Comment: Two injections of vaccine 1, given at intervals of five days, failed to protect the volunteer when tested one week following the second injection.

DISCUSSION

The negative results obtained in these preliminary experimental attempts to vaccinate susceptible volunteers with noninfectious filtrates obtained from suspensions of recently killed infective mosquitoes, with suspensions of dried infective blood kept for different periods of time, and with vaccine 1 prepared from infective mosquitoes, do not indicate that an effective prophylactic vaccine may not be developed. Because of the invisibility of the virus of dengue, it is impossible to estimate its concentration in the various materials used, or to determine whether it is killed or attenuated. The noninfective Mandler and Berkefeld filtrates, obtained from saline and broth suspensions of macerated infective mosquitoes, probably did not contain infective virus and, as would be expected, injections of this material failed to protect any of the individuals against subsequent infection. The desiccated blood, collected from experimental cases of dengue and frozen immediately, was kept for periods of four to seventy-nine days before use. The fact that this material failed to cause dengue indicates that the virus was either dead or attenuated. While these injections failed to prevent subsequent infection, still some of the volunteers had rather mild cases of dengue. In the vaccine (No. 1) prepared from a saline suspension of infectious mosquitoes, the virus was probably killed, although it may have been attenuated. Used in doses of 1 cubic centimeter, this vaccine failed to immunize volunteers when tested within two weeks after the injections had been given.

The recently reported work of Blanc and Caminopetros(1) (1929) concerning dengue vaccination is of interest. These investigators state that injections of dengue virus, killed by heat or by chemicals, had no protective value against infection with dengue. Therefore, they attempted to obtain an attenuated living virus that might give rise to a low-grade "symptomless" dengue, with subsequent immunity. It was found that dengue virus in blood could be attenuated by mixing it for five minutes with one-twentieth of its volume of bile, while the virus was killed by similar exposure to one-fifteenth of its volume of bile. As it was found that volunteers inoculated with the bile-virus mixture (1 to 15) were no longer susceptible to injections with bile-virus mixtures (1 to 20), these materials were combined and used as a vaccine. Twenty-two persons were doubly inoculated, first with the bile virus (1 to 15) and later with the bile virus (1 to 20); and when tested for immunity three weeks later only two of the individuals developed dengue.

The desirability of an attenuated virus for use as a prophylactic vaccine has also been recognized by the authors, who have considered the possibility of obtaining a constant supply by passage of the virus in series through mosquitoes. However, although there appeared to be some decrease in virulence of the virus after the second passage from mosquito to mosquito, this observation has not yet been confirmed; and consequently there has been no opportunity to attempt to immunize susceptible persons in this manner.

The possibility of developing an effective prophylactic vaccine is considered sufficiently promising to warrant further work on the subject. The development of such a vaccine, suitable for practical use in the immunization of troops before arrival in the Philippines, should make it possible to greatly reduce the incidence of this disease, which is one of the most important causes for loss of time among the military personnel of the Islands.

SUMMARY AND CONCLUSIONS

1. Subcutaneous inoculations of volunteers with filtrates obtained from saline suspensions of macerated infective mosquitoes failed to protect them against subsequent infection with dengue virus.
2. Subcutaneous inoculation of volunteers with saline suspensions of noninfective dried blood, previously collected from experimental cases, failed to protect against subsequent infection with dengue virus.
3. Inoculations with a vaccine prepared from infective *A. ægypti* also failed to produce protective immunity in man within periods of twelve days.
4. It is believed that an effective prophylactic vaccine against dengue may be developed by further investigation.

REFERENCE

1. BLANC, G., and J. CAMINOPETROS, Bull. Acad. Med. July 9, year 93, 3d Ser. 102 (1929) 26, 37–40 (Pasteur Inst. Athens). [Abstr. Trop. Dis. Bull. London 27 (1930) 2, 146.]

DENGUE IN LOWER ANIMALS

I. PHYSICAL SIGNS, TEMPERATURE, AND LEUKOCYTE REACTIONS IN ANIMALS INOCULATED WITH DENGUE VIRUS

The need for a diagnostic laboratory animal suitable for use in experimental studies of dengue led to the following investigation, in which attempts were made to produce dengue in chickens, lizards, guinea pigs, rabbits, goats, and monkeys.

While certain early observers, including Sandwith(1) and Hirsh,(2) reported epizoötics in birds, mice, rats, cats, dogs, and cattle that occurred coincident with outbreaks of dengue in man, investigators who have since attempted to infect lower animals experimentally have usually reported negative or inconclusive results. In 1914 Lavinder and Francis(3) announced experiments in which they inoculated nine *Macacus rhesus* monkeys with blood from dengue patients. In two tests the blood was collected on the second and third days of dengue, but in the other seven tests it was drawn on the third, fourth, or fifth day of the disease, at which time the virus is usually not transferable. Their conclusions were as follows:

> If dengue is conveyable to the rhesus monkey by intravenous or subcutaneous inoculation of defibrinated blood, these animals do not show the disease by changes in temperature, appearance of skin eruption or any of the ordinary symptoms of illness; further, that it is doubtful whether they show any definite and characteristic change in the white cell count, but the results obtained by us are perhaps sufficiently suggestive to invite further effort.

Kraus(4) in 1916 injected blood obtained from dengue patients into guinea pigs which were observed for subsequent periods of eight days. Neither fever nor other symptoms of infection appeared during this time. In 1917 the Japanese investigators Koizumi, Yamaguchi, and Tonomura(5) reported the results of animal experiments conducted in Formosa. They were unable to produce recognizable infections by the inoculation of small amounts of defibrinated blood from dengue patients into white mice, rabbits, dogs, and long-tailed Formosan monkeys. However, guinea pigs inoculated similarly became ill and usually died within seven to thirty-six days. By subinoculation from animals injected fifteen days previously similar symptoms were produced in other guinea pigs, which died within five to nineteen days if inoculated subcutaneously or intraperitoneally, and within twenty-eight to thirty-four days if the inoculation was made intravenously. They were unable to pass the infection through a third series of animals. It is unfortunate that the specific nature of the infection in these guinea pigs was not established.

Cleland, Bradley, and McDonald(6) in 1919 also reported experimental attempts to produce dengue in guinea pigs and rabbits during which they were unable to observe any signs of infection. In one experiment a guinea pig, injected with blood which produced dengue in a volunteer, was tapped at the end of seven and one-half days and 2 cubic centimeters of its blood was injected into a volunteer with negative results. Chandler and Rice(7) in 1923 attempted to infect guinea pigs, white mice, and a young rhesus monkey with dengue, but the results were considered negative. They also made the following observations:

> Furthermore, 65 guinea pigs of all ages were housed in an outdoor pen during the entire summer and fall where yellow fever mosquitoes swarmed. Only one became ill and three days later died from a respiratory infection. These pigs were freely bitten, even tormented by the mosquitoes and the percentage of dengue in that district was high.

They concluded "that guinea pigs are not susceptible to dengue organisms." The rhesus monkey, which they inoculated with about 5 cubic centimeters of citrated dengue blood drawn on the third day of the disease, appeared "chilly and morose" on the third and fourth days after inoculation, but there was no significant rise in temperature. On the fifth day he showed a fine papular rash on the chin and throat which was not considered to be a manifestation of dengue. These workers stated, "we are inclined to think that the inoculation had negative results as did those of Lavinder and Francis (1914); at any rate it was very doubtfully positive." Holt(8) in 1923 failed to produce recognizable evidence of infection in guinea pigs and rabbits inoculated with blood from dengue patients. Armstrong(9) (1923) attempted to convey dengue to guinea pigs, rabbits, white rats, and rhesus monkeys, but with negative results. He stated—

> Animals were injected with blood taken at various stages of the disease (5th hour to convalescence). The animals were observed for from 8 to 30 days in various cases. In no instance was the behavior of the injected

animals different from that of the controls. Blood counts were made in monkeys, rabbits and rats but no variations deemed significant were noted. In the case of the guinea pigs, however, the results were complicated by an epidemic of broncho-pneumonia which broke out among them after about the 15th day. Controls and injected animals were alike attacked.

Harris and Duval(10) in 1924 reported extensive investigations with guinea pigs and claimed to have transmitted dengue through a long series of these animals. However, many of the experimental animals were infected by organisms of the paratyphoid group, and they apparently failed to prove that the reactions described were due to the virus of dengue. Siler, Hall, and Hitchens(11) in 1926, in Manila, injected each of two young hogs intravenously with 10 cubic centimeters of blood from two early cases of dengue. One week later the animals were bled, and the mixed blood injected in 10 cubic centimeter amounts into two other hogs. After one week the latter animals were bled and their blood was inoculated into two susceptible volunteers, who failed to develop dengue from this injection. Counts of leukocytes in the blood of the hogs showed no significant changes.

Mancussakis(12) (1929) reported that guinea pigs inoculated with three times the dose of dengue virus required to infect volunteers showed no recognizable reaction. Blanc, Caminopetros, and Manoussakis(13) (1929) inoculated monkeys, dogs, pigeons, fowls, white rats, rabbits, and guinea pigs but failed to note any febrile or other reactions. However, in two cases serum was taken from guinea pigs about the fourth day after inoculation and injected into human volunteers, both of whom developed dengue after an incubation period of four days. They were not able to transfer the infection through guinea pigs in series. Blanc, Caminopetros, Dumas, and Saenz(14) in 1929 inoculated blood containing dengue virus into several species of Asiatic and African monkeys—macaques, baboons, cercopithecs, and margabeys. None of these animals showed any temperature reaction or clinical signs of dengue. However, when the blood from a certain number of these monkeys was transferred to man, dengue fever developed after an incubation period of four to five days. Hence, the monkeys concerned must have suffered from a latent or nonapparent infection. They drew the following conclusions:

1. The macaque monkeys *Cynomolgus fascicularis* and *Cercopithecus callitrichus,* inoculated with blood from human dengue patients suffer from a latent infection. Their blood, which is non-virulent twenty-four hours after inoculation becomes virulent from the 5th to the 8th day. It is no

longer virulent at the 12th day. A single experiment with a baboon yielded a negative result. 2. Monkeys which have suffered with latent dengue develop an immunity lasting at least 50 days. 3. The blood of monkeys infected with dengue is virulent for man. Under experimental conditions it does not seem to be virulent for the monkey, in which neither infection nor immunity develops.

PRESENT INVESTIGATION

In the present study attempts were made to infect chickens, lizards, mice, rabbits, goats, and monkeys with dengue. It was considered advisable to use animals that had been obtained in localities free from dengue or had been protected from mosquitoes since birth, in order to avoid the possibility of selecting animals that had already become immune, because of previous naturally acquired infection. However, such "protected" animals were not always available, and many of the experiments were done with animals obtained from the Bureau of Science in Manila.

Many of the preliminary experiments were limited to observations on the temperature and leukocyte reactions before and after inoculations with materials known to contain infective dengue virus. In later experiments attempts were made to demonstrate infection in the inoculated animals regardless of symptoms, by feeding normal *Aëdes* mosquitoes on them and later testing the mosquitoes for infectivity by allowing them to feed on volunteers.

EXPERIMENTAL

1. CHICKENS

The chickens used experimentally were obtained in the city market in Manila and kept in the laboratory for about one week.

Experiment 1.—February, 1929. Two cockerels and four chicks were inoculated subcutaneously with blood containing infective dengue virus and with saline suspensions of infected mosquitoes. The daily temperature and leukocyte counts on the cockerels did not indicate infection; and all of the chickens remained normal in appearance.

Experiment 2.—August 31, 1929. Four cockerels, Nos. 3, 4, 5, and 6, were inoculated into the "wattle" with 0.5 cubic centimeter each of infective blood collected from an experimental case of dengue (A.V. 31–D) during the first day of fever. The daily temperature reactions and leukocyte counts failed to indicate any infection.

Groups of normal *A. ægypti* fed on the chickens three, four, and five days after the inoculations were combined and designated lot 55. Similar groups fed on the sixth, seventh, and eighth days were combined and called lot 56. These two lots, of mosquitoes Nos. 55 and 56, were tested September

26, when one hundred twenty and one hundred five, respectively, fed on a susceptible volunteer (A.V. 41-F) but failed to cause dengue.

Comment.—Chickens obtained from Manila, where dengue is endemic, failed to show any recognizable reactions, following inoculations with dengue virus. Normal *A. ægypti* fed daily on such chickens between the third and eighth days after inoculation failed to transmit dengue to human volunteers.

2. LIZARDS

Two young lizards, caught in a dwelling in Manila, were each inoculated subcutaneously August 31, 1929, with 0.5 cubic centimeter amounts of blood collected from an experimental dengue case (A.V. 31-D) during the first day of the disease. So far as could be observed by their appearance the lizards remained well. They were liberated about one month later.

3. WHITE MICE

Experiment 1.—December 17, 1928. Two white mice raised in an unscreened cage in the City of Manila, were each inoculated subcutaneously with 0.5 cubic centimeter amounts of infective blood from a case of dengue (Keller). These animals showed no physical signs of illness.

Experiment 2.—January 9, 1929. Two white mice were each inoculated intraperitoneally with 0.5 cubic centimeter amounts of infective blood from A.V. 2-M. Two other mice were injected similarly with blood from A.V. 1-H. These mice failed to show any physical signs of illness.

Comment.—Subcutaneous and intraperitoneal inoculations of infective blood from experimental cases of dengue into white mice raised in an unscreened cage in the City of Manila, failed to cause any recognizable illness.

4. RABBITS

The rabbits used were obtained from an unscreened animal house in the Bureau of Science, Manila, and had, therefore, probably been exposed to the bites of dengue-infected mosquitoes for indefinite periods of time.

Experiment 1.—December 17, 1928. Two rabbits each received subconjunctival inoculations, into the left eye, of 0.1 cubic centimeters amounts of infective serum from a proved case of dengue (Keller). As a control the left eye of each rabbit was inoculated similarly with serum from a patient suffering with pneumococcus septicæmia. Neither of the animals showed any significant reaction during two weeks observation.

Experiment 2.—February 7, 1929. A rabbit (No. 1) was inoculated intracranially with 0.25 cubic centimeter of a suspension made by macerating two infective *A. ægypti*, lot 1, and two of lot 2, in 1.5 cubic centimeters of physiologic salt solution. After inoculation the rabbit showed a slight decrease in rectal temperature until the fourth day, after which it rose and remained at about the height noted on the day of inoculation until the tenth day when it reached 104° F. The leukocyte curve dropped gradually from 8,500 on the day of inoculation to 5,600 on the ninth day; and there was an abrupt drop in the neutrophilic granulocytes on the fifth, sixth, and seventh days. On the seventh day the hind quarters of the rabbit became paralyzed. The animal was killed and examined February 19. There was no evidence of infection and the paralysis was attributed to traumatism due to the method of inoculation.

TABLE 27.—*Rabbit 1 (gray rabbit).*

	Days after subdural inoculation with dengue-infected mosquitoes.												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Temperature °F	103.0	102.4	101.6	101.8	101.6	103.2	103.4	103.0	103.2	103.6	104.0	104.0	104.6
Total leukocytes	8,500	9,600	8,200	9,100	8,000	7,900	7,600	7,800	6,500	5,600	7,500	13,300	9,400
Neutrophiles	3,800	5,800	3,900	3,700	3,000	1,300	1,200	1,700	2,300	2,300	2,500	5,700	3,100
Lymphocytes	5,000	3,600	4,100	4,600	4,600	6,100	5,000	5,300	3,700	3,000	4,700	6,500	5,000
Monocytes	250	200	250	270	480.0	320	1,400	630	460	50	320	900	1,800

As a control on this experiment, another normal rabbit (No. 1–A) was inoculated similarly with a suspension of normal mosquitoes. This animal also showed irregular daily temperature and leukocyte reactions, as indicated in Table 28.

TABLE 28.—*Rabbit 1–A.*

	Days after subdural inoculation with normal mosquitoes.												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Temperature °F	103.8	101.2	101.0	101.4	102.1	102.6	104.0	101.2	101.0	104.0	102.8	103.7	102.5
Total leukocytes	7,900	10,000	7,900	8,500	7,700	7,800	13,300	7,500	8,900	8,500	7,500	10,700	8,300
Neutrophiles	4,200	6,500	2,300	3,200	1,600	2,400	2,500	1,300	1,200	680	1,400	1,300	1,450
Lymphocytes	3,700	3,800	5,300	4,700	4,700	5,000	7,000	5,700	6,200	6,600	4,100	7,850	5,600
Monocytes	150	300	240	670	1,400	460	3,200	530	1,300	1,200	910	1,500	1,400

Experiment 3.—February 19, 1929. An adult rabbit (No. 2), which since February 9 had received daily intravenous inoculations with 3 cubic centimeters of a 3 per cent suspension of Higgins' India ink in physiological salt solution, was inoculated intracranially with 0.2 cubic centimeter of a suspension prepared by macerating three infective *A. ægypti* in 1.5 cubic centimeters of salt solution. The daily temperature and leukocyte reactions are shown in Table 29.

TABLE 29.—*Rabbit 2 (dark gray).*

	Days after subdural inoculation with dengue-infected mosquitoes.													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Temperature ....°F..	103.0	102.4	102.2	103.2	102.4	102.3	102.0	102.2	102.2	102.2	....	102.2	103.0	101.8
Total leukocytes......	8,100	8,200	5,900	6,100	5,600	6,500	7,500	8,150	8,200	7,700	....	8,100	9,000	9,200
Neutrophiles..........	1,500	4,000	2,750	1,700	1,750	1,600	2,600	2,600	2,400	1,600	....	2,700	990	1,600
Lymphocytes.........	5,800	3,600	2,500	3,500	4,300	3,100	4,200	5,100	5,300	5,200	....	4,900	3,450	3,200
Monocytes...........	490	410	600	800	230	1,300	300	250	320	530	....	320	540	280

As a control another rabbit (No. 2-A), similarly prepared, was inoculated with a suspension of normal *A. ægypti*, with the results indicated in Table 30.

TABLE 30.—*Rabbit 2-A (light gray).*

	Days after subdural inoculation with normal mosquitoes.													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Temperature ....°F..	102.3	102.8	101.8	102.2	101.2	102.0	101.8	102.4	102.0	101.8	....	101.8	102.2	101.6
Total leukocytes......	7,800	7,600	10,200	7,400	6,200	6,200	8,700	9,700	7,900	8,100	....	7,200	7,900	8,200
Neutrophiles..........	1,100	1,200	810	2,400	1,600	1,200	860	1,700	1,200	2,200	....	2,000	1,200	1,200
Lymphocytes.........	6,300	5,300	6,700	4,700	5,000	5,200	6,950	6,800	5,950	5,500	....	4,900	5,900	6,650
Monocytes...........	400	990	2,500	230	190	200	690	180	545	485	....	150	510	320

Experiment 4.—February 25, 1929. A normal rabbit (No. 3) was inoculated intracranially with 0.25 cubic centimeter of infective blood from an experimental case of dengue (A.V. 3–A). The temperature and leukocyte reactions of this rabbit are shown in Table 31.

TABLE 31.—*Rabbit 3 (red rabbit).*

	Days after subdural inoculation with 0.2 cc dengue-infected blood.											
	0	1	2	3	4	5	6	7	8	9	10	11
Temperature °F	....	103.8	101.6	102.3	....	103.0	103.4	102.6	103.0	102.4	102.8	102.6
Total leukocytes	15,800	12,100	15,500	15,300	....	13,800	14,000	18,000	11,500	13,000	14,800	13,300
Neutrophiles	4,000	2,100	8,100	6,600	....	2,500	2,550	3,500	1,900	2,200	3,300	3,100
Lymphocytes	10,700	9,400	4,300	8,000	....	10,800	10,700	14,200	8,900	9,900	11,300	9,500
Monocytes	1,100	360	2,000	760	....	560	850	365	450	520	455	540

Experiment 5.—May 11, 1929. Two normal rabbits (Nos. 4 and 5) were each inoculated intracranially with 0.5 cubic centimeter amounts of citrated blood from *Macacus philippinensis* 16. May 15 the rabbits were again inoculated similarly with 0.25 cubic centimeter of infective blood from an experimental case of dengue (A.V. 9–D). Rabbit 4 died immediately as a result of the inoculation. The daily temperature and leukocyte changes in rabbit 5 are shown in Table 32.

COMMENT

Subconjunctival and intracranial inoculations of dengue virus into these rabbits, which had previously lived unscreened in the City of Manila, failed to produce any specific diagnostic evidence of infection with dengue.

5. GOAT

April 16, 1929, a young male goat, obtained from the Bureau of Science farm, was inoculated intratesticularly with 0.25 cubic centimeter of infective blood from experimental case of dengue A.V. 6–R. No significant local or general reaction was noted. The rectal temperature showed no rise. The total leukocyte counts remained between 13,000 and 17,800. However, there was a marked decrease in neutrophiles on the fifth and sixth days following inoculation. (See Table 33). Comment: This animal failed to show any significant evidence of infection with dengue.

TABLE 32.—*Rabbit 5.*

	Days after subdural inoculation with 0.5 cc blood from monkey 16.				
	0	1	2	3	4
Temperature °F	102.6	102.2	102.6	102.0	105.0
Total leukocytes	7,900	8,550	6,550	6,800	7,350
Neutrophiles	1,975	1,282.5	931	1,836	2,058
Lymphocytes	5,927	7,267.5	5,453	4,896	5,292

	Days after subdural inoculation with 0.5 cc blood from monkey 16.				
	5	6	7	8	9
Temperature °F	104.0	104.0	102.8	102.0	101.8
Total leukocytes	6,950	10,150	7,400	8,050	9,300
Neutrophiles	1,598.5	2,030	518	885.5	1,023
Lymphocytes	5,351.5	8,120	7,084	8,277	----------

TABLE 33.—*Goat 1.*

	Days after inoculation with 0.25 cc dengue blood.				
	1	2	3	4	5
Temperature °F	102.2	102.2	101.8	102.0	101.8
Total leukocytes	13,900	13,550	13,000	15,500	14,350
Neutrophiles per cent	48	25	39	27	34
Lymphocytes do	35	63	50	65	57
Monocytes do	6	2	4	2	1

	Days after inoculation with 0.25 cc dengue blood.				
	6	7	8	9	10
Temperature °F	101.0	101.6	101.2	100.0	101.0
Total leukocytes	15,650	13,450	14,900	17,850	17,850
Neutrophiles per cent	19	22	51	40	54
Lymphocytes do	78	69	38	56	41
Monocytes do	1	4	2	1	4

6. GUINEA PIGS

The guinea pigs used in the following experiments had previously been kept in an unscreened animal house in the City of Manila and had no doubt been bitten many times by mosquitoes.

Experiment 1.—April 16, 1929. Guinea pig 1 received an intratesticular injection of 0.25 cubic centimeter of defibrinated blood, drawn from A.V. 6-R during his first day of dengue. April 22, 1929, the animal was

TABLE 32.—*Rabbit 5.*

	Days after subdural inoculation with 0.5 cc blood from monkey 16.									
	0	1	2	3	4	5	6	7	8	9
Temperature °F.	102.6	102.2	102.6	102.0	105.0	104.0	104.0	102.8	102.0	101.8
Total leukocytes	7,900	8,550	6,550	6,800	7,350	6,950	10,150	7,400	8,050	9,300
Neutrophiles	1,975	1,282.5	931	1,836	2,058	1,598.5	2,030	518	885.5	1,023
Lymphocytes	5,927	7,267.5	5,453	4,896	5,292	5,351.5	8,120	7,084	8,277	----------

TABLE 33.—*Goat 1.*

	Days after inoculation with 0.25 cc dengue blood.									
	1	2	3	4	5	6	7	8	9	10
Temperature °F.	102.2	102.2	101.8	102.0	101.8	101.0	101.6	101.2	100.0	101.0
Total leukocytes	13,900	13,550	13,000	15,500	14,350	15,650	13,450	14,900	17,850	17,850
Neutrophiles per cent.	48	25	39	27	34	19	22	51	40	54
Lymphocytes do.	35	63	50	65	57	78	69	38	56	41
Monocytes do.	6	2	4	2	1	1	4	2	1	4

6. GUINEA PIGS

The guinea pigs used in the following experiments had previously been kept in an unscreened animal house in the City of Manila and had no doubt been bitten many times by mosquitoes.

Experiment 1.—April 16, 1929. Guinea pig 1 received an intratesticular injection of 0.25 cubic centimeter of defibrinated blood, drawn from A.V. 6–R during his first day of dengue. April 22, 1929, the animal was sacrificed and examined. There was no gross evidence of infection of the testicle and no diagnostic sign of dengue. (See Table 34).

Experiment 2.—May 15, 1929. Guinea pig 2 received intratesticularly 1 cubic centimeter of citrated blood obtained from A.V. 9–D during the first twenty-four hours of dengue fever. May 15 to May 28, no evidence of general reaction or of pathological change in the testicle. May 28, discarded. (See Table 35.) There was no indication that dengue occurred in this animal.

Experiment 3.—May 15, 1929. Guinea pig 3 received intratesticularly 1 cubic centimeter of citrated blood obtained from A.V. 9–D during the first twenty-four hours of experimental dengue fever. May 18 fed eighty-eight *Aëdes ægypti*, designated as lot 21 (see record of *A. ægypti*). (This lot of mosquitoes was tested for infectivity June 5, 1929, by feeding forty-five of them on a susceptible volunteer (A.V. 14–A), but dengue did not develop.) (See Table 35.) The guinea pig was then sacrificed and examined, but no evidence of pathology was noted. One cubic centimeter of blood from the heart was injected into the testicle of guinea pig 6. The testicle was removed, emulsified in 0.85 cubic centimeter of saline, filtered through a preliminary Mandler filter, which retained *M. melitensis*, and 0.5 cubic centimeter of the filtrate was injected subcutaneously into guinea pigs 7 and 8. No evidence was obtained to indicate that guinea pig 3 had dengue.

Experiment 4.—May 18, 1929. Guinea pig 4 was injected intratesticularly with 0.5 cubic centimeter of citrated blood from A.V. 12–S, who was in the first twenty-four hours of dengue fever. May 18 to 30, 1929, no evidence of infection. (See Table 35.) Testicle showed no enlargement. Lymph glands not palpable. May 30, 1929, discarded. There was no evidence that dengue occurred in this animal.

Experiment 5.—May 18, 1929. Guinea pig 5 was injected intratesticularly with 0.5 cubic centimeter of citrated blood obtained from A.V. 12–S during the first twenty-four hours of dengue fever. May 18 to 30, not sick. Testicles and lymph glands normal. Slight leukopenia was noted on the fifth day. May 30, discarded. This animal showed no significant evidence of infection with dengue. (See Table 35.)

Experiment 6.—May 18, 1929. Guinea pig 6 was injected intratesticularly with 1 cubic centimeter of blood from the heart of guinea pig 3. (Guinea pig 3 showed leukopenia this date and was sacrificed for examination.) May 18 to 30, no evidence of illness; testicles and lymph glands normal. (See Table 35.) May 30, discarded. This animal showed no significant evidence of dengue.

Experiment 7.—May 18, 1929. Guinea pig 7 was injected intratesticularly with 0.5 cubic centimeter of filtrate obtained by passing an emulsion of testicle from guinea pig 3 through a preliminary type of Mandler filter. May 22, 1929, normal; no change in testicles and no enlargement of inguinal lymph nodes. May 23, 1929, slight leukopenia developed, but the animal was apparently normal. (See Table 35.) Sacrificed for autopsy and to obtain material for injection into guinea pigs 11, 12, and 13. See protocols. No evidence of pathology noted. This animal showed no significant evidence of dengue.

Experiment 8.—May 18, 1929. Guinea pig 8 received an intratesticular injection of 0.5 cubic centimeter of a filtrate obtained by passing an emulsion of the testicle of guinea pig 3 through a preliminary type of Mandler filter. (Guinea pig 3 had a leukopenia May 18, but was apparently not sick.) May 19 to 30, no evidence of testicular change, enlargement of inguinal lymph nodes, or of illness; no leukopenia. (See Table 35.) May 30, discarded. This animal showed no significant evidence of dengue.

Experiment 9.—May 23, 1929. Guinea pig 9 was injected intratesticularly with 0.75 cubic centimeter of citrated blood obtained from A.V. 13–W, during the first twenty-four hours of dengue fever. May 24 to June 2, no evidence of testicular change, glandular enlargement, or of illness. (See Table 35.) June 2, discarded. This animal showed no significant evidence of dengue.

Experiment 10.—May 23, 1929. Guinea pig 10 was injected intratesticularly with 0.75 cubic centimeter of citrated blood, obtained from A.V. 13–W, during the first twenty-four hours of dengue. May 24 to June 5, no evidence of reaction in testicle or enlargement of lymph glands; there was no significant indication of illness. (See Table 35.)

Experiment 11.—May 23, 1929. Guinea pig 11 was injected intratesticularly with 0.75 cubic centimeter of blood from the heart of guinea pig 7, which showed a slight leukopenia on this date. May 24 to June 4, no evidence of illness or leukopenia (see Table 35); or of reaction in the testicle or lymph glands. June 5, discarded.

Experiment 12.—May 23, 1929. Guinea pig 12 was injected intratesticularly with 0.5 cubic centimeter of a saline emulsion of the testicle from guinea pig 7, which showed a slight leukopenia on this date but was otherwise normal. May 24 to June 5, no change in the testicle or in the lymph glands. The animal appeared well and there was no leukopenia. (See Table 35.) This animal showed no evidence of infection.

Experiment 13.—May 23, 1929. Guinea pig 13 was injected intratesticularly with 0.5 cubic centimeter of an emulsion of testicle removed from guinea pig 7. May 24 to June 5, no evidence of illness, testicular change, or of enlargement of the lymph glands. May 27, the fifth day after the inoculation, there was a decrease in leukocytes, but as this also occurred June 5, it must be discounted. This animal showed no evidence of infection.

An attempt was made to obtain nonimmune guinea pigs in the following manner: Pregnant female guinea pigs were kept in mosquito-proof cages for at least a week before they gave birth to young. The young were kept in the screened cages until used experimentally.

Experiment 14.—February 28, 1930, 11 a. m. Guinea pig 14-A, born sixteen days before and kept in a screened cage, was bitten by seven *Aëdes ægypti* of lot 135. This lot of mosquitoes had been infected December 13, 1929 (seventy-seven days previously) from A.V. 61–J. During thirteen days observation, from February 28 to March 13, there was no evidence of illness and the daily temperature records and leukocyte counts were not considered significant of infection. In order to test for a possible symptomless dengue infection, normal *Aëdes ægypti* were fed as follows: March 4, fed twenty-six; March 5, fed twenty-two; March 6, fed twenty-eight; March 7 fed thirty-two, and March 8, fed twenty-six.

These mosquitoes were combined, designated lot 175, and on March 20, 1930, one hundred two fed on a susceptible volunteer (A.V. 89–S). This volunteer did not develop dengue and he was later proved susceptible to infection by known infected mosquitoes. March 23, 1930, the guinea pig died. Comment: The feeding of dengue infected *Aëdes ægypti* on a sixteen-day-old guinea pig, which for the duration of its life had been screened from mosquitoes, did not cause infection with dengue. It was considered possible that the age of the animal, 16 days, might account for its apparent immunity.

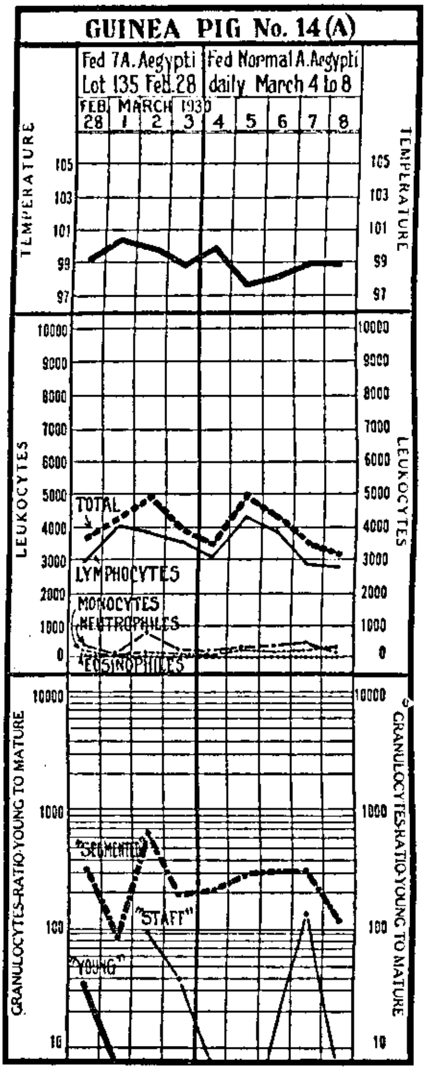

FIG. 50. Experimental dengue in guinea pig 14–A.

Experiment 15.—March 19, 1930. Guinea pig 15–B, which had been born fifteen days previously and kept in a screened cage, was bitten by twelve *A. ægypti* of lot 165. This lot of mosquitoes had been potentially infected thirty-nine days previously by feeding on A.V. 76–M, during the first day of dengue, and had subsequently transmitted dengue to A.V. 86–B. From March 19 to April 3 the guinea pig remained normal in appearance. The daily temperature records and leukocyte counts are shown in the accompanying chart (fig. 51). The latter record is suggestive of dengue infection. In order to determine the possibility of the development of a symptomless infection, normal *A. ægypti* were fed as follows: March 24, thirty-two fed; March 25, thirty-two fed; March 26, twenty-one fed, and March 27, twenty fed. These mosquitoes were combined and designated lot 187. April 23, 1930, twelve mosquitoes of this lot fed on an Ilocano volunteer, F.V. 34–R, at Baguio, Mountain Province, but dengue did not follow. The susceptibility of this volunteer was not determined, but as he had lived in the lowlands he was probably immune. April 29, 1930, four mosquitoes of lot 187 fed, and April 30 eight fed on volunteer A.V. 98–R but did not cause dengue. This volunteer was subsequently proved to be immune. Comment: The first volunteer used, F.V. 34-R, being a lowland

TABLE 34.—*Attempts to produce dengue in guinea pigs.*

Inoculation with dengue virus.					Result of inoculation.					
Guinea pig No.	Source of infective material.	Date.	Amount.	Site of injection.	Under observation.	Fever.	Leukopenia.	Other signs of illness.	Reaction at site of injection.	Conclusions.
			cc.		*Days.*					
1	A.V. 6-R	Mar. 16, 1929	0.25	Testicle	6	None	Slight, 2d day	None	None	Negative.
2	A.V. 9-D	May 15, 1929	1.0	do	12	do	None	do	do	Do.
3	A.V. 9-D	May 15, 1929	1.0	do	[a] 3	do	Yes, 2d and 3d days	do	do	(?)
4	A.V. 12-S	May 18, 1929	0.5	do	13	do	None	do	do	Negative.
5	A.V. 12-S	May 18, 1929	0.5	do	11	do	Slight, 5th day	do	do	(?)
6	Blood, guinea pig 3	May 18, 1929	1.0	do	12	do	None	do	do	Negative.
7	Testicle, guinea pig 3	May 18, 1929	0.5	do	[b] 5	do	Slight	do	do	(?)
8	do	May 18, 1929	0.5	do	11	do	None	do	do	Negative.
9	A.V. 13-W	May 23, 1929	0.75	do	9	do	do	do	do	Do.
10	A.V. 13-W	May 23, 1929	0.75	do	12	do	do	do	do	Do.
11	Blood, guinea pig 7	May 23, 1929	0.75	do	13	do	Slight, 2d day	do	do	(?)
12	Testicle, guinea pig 7	May 23, 1929	0.5	do	13	do	Slight, 2d and 3d days	do	do	(?)
13	do	May 23, 1929	0.5	do	13	do	None	do	do	Negative.

[a] Sacrificed at end of third day for transfer of material to guinea pigs 6, 7, and 8.

[b] Sacrificed the fifth day for transfer of material to guinea pigs 12 and 13.

TABLE 35.—*Total leukocytes and temperature records of guinea pigs following inoculations of dengue virus.*

Guinea pig No.	Blood count and temperature.	Days following inoculation.							
		1	2	3	4	5	6	7	8
1	Total W.B.C.	9,750	7,250	5,600	11,450	6,400	7,100	----	----
	Temperature °F	99.2	99.2	100.6	100	99	99.8	----	----
2	Total W.B.C.	9,600	5,400	7,000	8,300	11,750	14,850	20,200	11,600
	Temperature °F	97.8	99.8	100.6	98.2	99.8	99.2	99.2	98.6
3	Total W.B.C.	9,400	3,900	3,650	----	----	----	----	----
	Temperature °F	102.4	101.2	102.8	----	----	----	----	----
4	Total W.B.C.	5,250	6,150	7,950	7,200	9,500	8,100	8,300	7,800
	Temperature °F	101	101	100.4	98.6	100	100.2	99.8	101
5	Total W.B.C.	8,100	6,400	8,050	7,100	5,300	5,600	9,350	9,850
	Temperature °F	98.8	100	101.8	101.4	100	100.6	99.6	101
6	Total W.B.C.	4,800	7,350	7,800	6,300	10,150	8,650	8,700	6,950
	Temperature °F	98.4	99.8	99.4	101.4	99.6	98.6	99	100.6
7	Total W.B.C.	7,250	9,450	7,900	6,850	5,050	----	----	----
	Temperature °F	100	98.8	101	100.8	100.2	----	----	----
8	Total W.B.C.	10,550	9,550	9,600	15,300	7,850	8,200	8,050	11,650
	Temperature °F	100.2	101	101.2	101	100.4	101.2	99.8	100.6
9	Total W.B.C.	10,300	10,300	11,950	10,000	[illegible],200	8,800	8,250	6,850
	Temperature °F	100.6	99.4	101	101	100.6	101	101	99
10	Total W.B.C.	8,150	12,100	7,750	8,600	8,200	11,700	7,600	9,750
	Temperature °F	101.2	98	100.4	100.8	101.8	101.4	101.8	101.6
11	Total W.B.C.	9,300	5,150	7,200	8,650	14,800	11,800	13,600	8,350
	Temperature °F	101.8	100.8	101.6	103.4	99.6	100.6	100.8	101
12	Total W.B.C.	9,400	5,500	5,000	24,400	7,950	11,450	12,200	15,500
	Temperature °F	102.2	101.4	102	100.8	100.8	101	101	100.8
13	Total W.B.C.	16,350	13,900	12,250	9,150	6,350	9,400	8,400	9,900
	Temperature °F	100	100.2	100.2	99.2	99.8	102	101.2	100.8
14	Total W.B.C.	3,700	4,200	4,800	3,950	3,400	4,800	4,350	3,500
	Temperature °F	99.2	100.4	99.8	98.8	99.6	97.6	89.2	98.8

Guinea pig No.	Blood count and temperature.	Days following inoculation.							
		9	10	11	12	13	14	15	16
1	Total W.B.C.								
	Temperature °F								
2	Total W.B.C.	8,950	10,000	13,600					
	Temperature °F	98.6	99	97					
3	Total W.B.C.								
	Temperature °F								
4	Total W.B.C.	12,600	9,950	22,850	12,100	23,650	14,500	13,100	13,910
	Temperature °F	99	100.4	101.8	101	101	101.2	102.2	102.4
5	Total W.B.C.	13,350	7,150	11,700					
	Temperature °F	100.8	101.8	101.4					
6	Total W.B.C.	7,650	8,350	5,400					
	Temperature °F	99	99.6	101.6					
7	Total W.B.C.								
	Temperature °F								
8	Total W.B.C.	9,150	14,900	10,250					
	Temperature °F	100	101.2	101					
9	Total W.B.C.								
	Temperature °F	99.8							
10	Total W.B.C.	10,300	9,400	11,500	7,000				
	Temperature °F	102.4	100.2	100.8	98.6				
11	Total W.B.C.	8,650	9,650	7,900	11,550	8,750	11,550	11,550	
	Temperature °F	100.6	100.8	100.4	100.8	100.6	101.2	100.6	
12	Total W.B.C.	16,200	9,300	8,750	8,550	6,350	9,300	7,100	
	Temperature °F	100.4	101.4	100.4	101	102.8	101	101.4	
13	Total W.B.C.	10,800	11,350	8,100	7,800				
	Temperature °F	99.2	100.2	99.6	100.6				
14	Total W.B.C.	3,100							
	Temperature °F	98.8							

Filipino, was probably immune. The second volunteer, A.V. 98-R, was immune. Since both of the volunteers were immune, no conclusion could be drawn relative to the infection of guinea pig 15-B.

Experiment 16.—March 19, 1930. Guinea pig 16-C, that had been born in a screened cage and kept there for fifteen days, was bitten by ten known infected *A. albopictus* of lot 160. This guinea pig showed no symptoms of infection during the following two weeks.

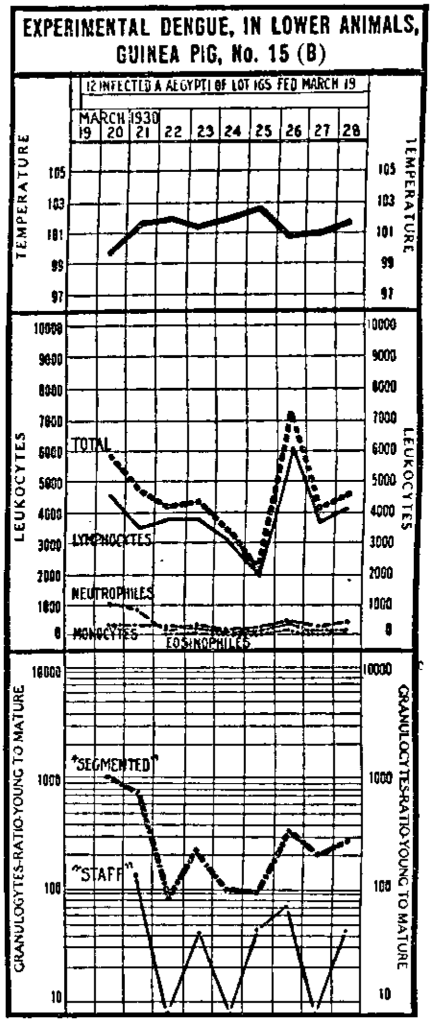

Fig. 51. Experimental dengue in guinea pig 15-B.

The temperature and leukocyte counts are shown in fig. 52. The leukocyte curve suggested dengue infection. Normal A. *ægypti* were fed on this guinea pig as follows: March 24, thirty-one fed; March 25, thirty-four fed; March 26, twenty fed, and March 27, twenty fed. These mosquitoes were combined and designated lot 188. April 23, 1930, twenty-five mosquitoes of lot 188 fed on an Ilocano volunteer, F.V. 30-M, at Baguio, Mountain Province, but failed to cause dengue. The susceptibility of F.V. 30-M was never determined, but as he had lived in the lowlands, he was probably immune. April 29, six fed, and April 30, twelve mosquitoes of lot 188 fed on a susceptible volunteer, A.V. 96-H, but did not cause dengue. Comment: The first volunteer used, F.V. 30-M, being a lowland Filipino, was probably immune. While guinea pig 16-C was only fifteen days old and might have been immune to dengue, the fact that only eighteen of the mosquitoes which took blood from it later fed on the American volunteer makes it impossible to conclude that the animal was not infected.

7. MONKEYS

The recent discovery that certain species of monkeys from nonendemic areas are susceptible to infection with the virus of yellow fever suggested the possibility that the case with dengue might be similar. Accordingly, five *Macacus rhesus*, which had lived in the National Zoölogical Park in Washington, D. C., for several years, including at least one animal born there, were obtained in May, 1928, through the courtesy of the director, Dr. W. M. Mann. These monkeys were placed in screened mosquito-proof cages and brought on an Army transport via Panama to Manila, where they arrived June 30, 1928. They were kept in screened cages at the Bureau of Science until used experimentally early in 1929. Later in 1929 arrangements were made, through Colonel Bunshiro Tanabe, M. C., Imperial Japanese Army, with an animal dealer in Tokyo for the purchase of two lots of Japanese monkeys—*Macacus fuscatus*. Five monkeys of the first shipment died en route, and the one animal that arrived safely in Manila was in an unscreened box. The four Japanese monkeys of the second shipment, which arrived several months later in good condition, were transported in screened mosquito-proof cages. As a control on the groups of *M. rhesus* (Audebert) (15) from Washington and *M. fuscatus* (Blythe) (15) from Tokyo, a large number of native Philippine monkeys, *Macacus philippinensis* S. Geoffroy (15) (*Cynomolgus philippinensis*), obtained both from endemic dengue areas, such as Manila, and from dengue-free localities

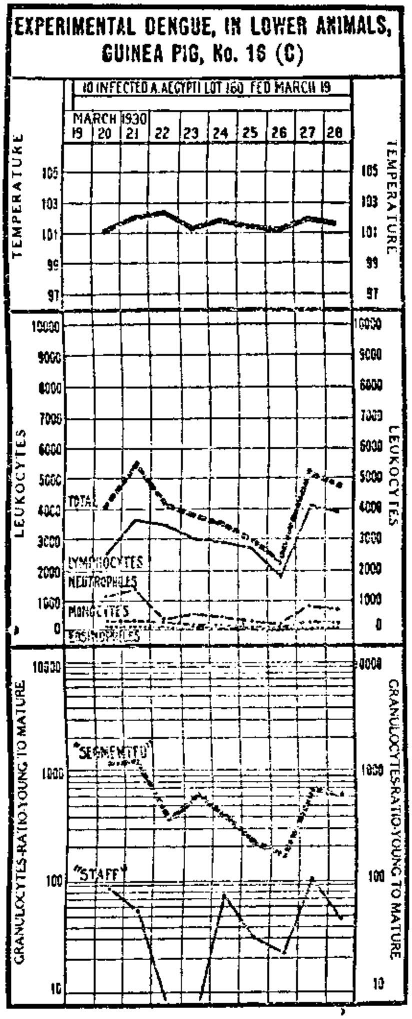

FIG. 52. Experimental dengue in guinea pig 16–C.

in the mountains of northern Luzon, were also used in transmission experiments.

Source of dengue virus.—In all of the experiments, excepting No. 13, the materials used in attempting to infect the monkeys were known to have contained dengue virus in amounts sufficient to infect man. The blood from dengue patients injected into the monkeys was usually drawn on the first day of the disease, never later than the second day; and in practically every instance its infectivity was proved by the fact that normal *A. ægypti*, fed on the patient at the time the blood was collected, later transmitted dengue to man. That the lots of mosquitoes fed on monkeys were infected was indicated by the fact that other mosquitoes of the same lots transmitted dengue to human volunteers.

Recognition of dengue infection in inoculated monkeys.—Previous studies of the striking leukocyte curves obtained in making daily counts of blood cells from a series of experimental cases of dengue in man, suggested the possibility that similar daily observations on monkeys, during the period following the inoculation of dengue virus, might serve as a means for the identification of the infection, should it occur in these animals. Consequently, in the earlier experiments with monkeys, the observations were limited almost entirely to daily studies of the physical appearance, temperature reactions, and leukocyte curves.

The temperature observations were made by rectum, usually about 11 a. m. with an ordinary clinical thermometer. Blood for the leukocyte counts was collected at the same time each day from a finger or toe. Experience in making several hundred leukocyte counts on normal monkeys indicated that a great variation is to be expected in the total leukocyte counts made at different times from the same animal. Therefore, in studying the results obtained in the transmission experiments, it was considered necessary to discount any but marked changes in the leukocyte counts. These observations were made on each animal used for a period of from two to ten days before the experimental inoculation.

In the later experiments the occurrence of infection was determined by feeding normal *Aëdes* on the monkeys each day during the period of possible infection and by subsequently testing these composite groups of mosquitoes for virus by feeding them on susceptible human volunteers.

2. OBSERVATIONS ON THE TEMPERATURE AND LEUKOCYTE REACTIONS OF MACACUS RHESUS, M. FUSCATUS, AND M. PHILIPPINENSIS, FOLLOWING THEIR INOCULATION WITH DENGUE VIRUS

MACACUS RHESUS

Experiment 1.—January 10, 1929. *Macacus rhesus* 2 from Washington, D. C., was bitten this date by four infected *A. ægypti* of lot 1, and January 11 by three more mosquitoes of the same lot. No physical signs of dengue were observed. The rectal temperature reactions were irregular. The total leukocyte count, which was 15,050 cells January 10, dropped to 10,400 January 13, then rose abruptly to 22,850 on the 14th, after which it returned to 8,000 January 17. The enormous increase in cells January 13 was due largely to a lymphocytosis, while on the other hand the neutrophiles decreased markedly January 13, 15, 16, and 17.

This monkey was tested again April 20, 1929, by the injection of 2 cubic centimeters of citrated blood from F.V. 18–P. The reaction was not significant.

Comment.—In these experiments the evidence was not considered sufficient to indicate whether or not this animal had developed dengue.

Experiment 2.—January 31, 1929. *Macacus rhesus* 3 from Washington, D. C., was bitten by fifteen infected *A. ægypti* of lot 3. No evidence of illness was recognized; both the temperature and leukocyte curves were irregular and within the normal range. Comment: In this experiment the evidence failed to indicate whether or not dengue had occurred.

Experiment 3.—January 31, 1929. *Macacus rhesus* 4 from Washington, D. C., was injected subcutaneously with 0.75 cubic centimeter of a suspension made by macerating twenty-two infected *A. ægypti* of lot 3 in 2 cubic centimeters of physiologic salt solution. There was no significant change in the daily temperature or leukocyte reactions following the injection. The lowest leukocyte count, 8,500, was obtained on the fifth day after injection.

Another attempt to infect this monkey was made April 20, 1929. With 2 cubic centimeters of citrated blood from F.V. 18–P. The reactions were not significant.

Comment.—In the experiment on this animal the evidence failed to indicate whether or not dengue had occurred.

Experiment 4.—February 28, 1929. *Macacus rhesus* 5 was brought to Manila in a screened cage from Washington, D. C., but escaped on arrival in Manila and spent one night unscreened before he was recaptured. February 28, 1929, this monkey was bitten by twelve infected *A. ægypti* of lot 3. As with the other four monkeys of this species, no significant diagnostic change was observed in the temperature or leukocyte curves. An enormous increase in neutrophilic granulocytes occurred from the fifth to the tenth day. Comment: In this experiment the evidence failed to indicate whether or not dengue had occurred.

MACACUS FUSCATUS

Experiment 5.—March 1, 1929. *Macacus fuscatus* 1, brought to Manila in an unscreened cage from Tokyo, Japan, was bitten by one infected

A. ægypti of lot 3, and again March 2 by two mosquitoes of lot 3 and by two of lot 2. This monkey showed an enormous increase in the numbers of lymphocytes, which rose from 4,770 March 1 to 12,560 March 7. Very little change was observed in the numbers of neutrophiles. Comment: This type of leukocyte reaction is the reverse of that observed during dengue in man. However, at the time of the experiment it was considered possible that it might represent a peculiar response of *M. fuscatus* to infection with dengue virus; and this led us to order another group of these monkeys from Japan for further experimental use. From later observations it is believed that the lymphocytosis observed in this monkey (No. 1) was probably due to causes other than dengue.

Note.—Later *M. fuscatus* 1 was inoculated intracardially with 2 cubic centimeters of citrated blood from a typical clinical case of dengue (F. V. 18–P). The temperature and leukocyte reactions following this inoculation were not considered significant.

MACACUS PHILIPPINENSIS

Experiment 6.—December 17, 1928 (noon). *Macacus philippinensis* 1, obtained in Manila, was injected subcutaneously with 1.5 cubic centimeters of citrated infected blood, which had just been collected from a case of dengue (Keller) during the second day of the disease. The infectivity of this blood was proved by the fact that *A. ægypti*, lot 1, which fed on Keller at the same time, later transmitted dengue to human volunteers. The monkey, however, failed to show any recognizable physical signs of infection and there was no striking change in the daily rectal temperature reactions. However, on the fourth, fifth, and sixth days after inoculation, there occurred a decrease in the number of leukocytes, particularly the neutrophilic granulocytes. This was accompanied by the appearance of from 4 to 6 per cent of the large vacuolated monocytes commonly found during or at the end of dengue in man. Comment: The decrease in leukocytes observed in this monkey occurred within the period usually required for the appearance of dengue in man; and while not proven to be diagnostic of the infection in this instance, the reaction was considered suggestive.

Experiment 7.—December 17, 1928 (noon). *Macacus philippinensis* 2, obtained in Manila, was inoculated subcutaneously with 1.5 cubic centimeters of citrated blood, which had just been collected from a dengue patient (Keller) during the second day of the disease. *Aëdes ægypti*, lot 1, fed on Keller at this time, later transmitted dengue to susceptible human volunteers. After inoculation the monkey was observed for two weeks, during which time there was no evidence of eruption or other physical signs of dengue. The daily rectal temperature fluctuations were within normal limits. However, there was a marked decrease in the leukocyte counts, from a preliminary count of 12,050 to about 5,000 cells on the fourth and fifth days after inoculation. This was due almost entirely to a decrease in the number of neutrophiles. Comment: The daily leukocyte curve of this monkey suggested the possibility that infection with dengue might have occurred about the fourth or the fifth day, but this was not proved.

Experiment 8.—January 10, 1929. *Macacus philippinensis* 4, obtained in Manila, was bitten by thirteen infected *A. ægypti* of lot 1. This animal showed no physical signs of illness. There was considerable variation in the daily leukocyte counts, but the curve was not considered indicative of dengue infection.

Experiment 9.—January 31, 1929. *Macacus philippinensis* 6, obtained in Manila, was bitten by ten infected *A. ægypti* of lot 3. The daily temperature variations were within normal limits, while the number of leukocytes increased steadily during the twelve days observation. There was no indication that this animal had developed dengue.

Experiment 10.—January 31, 1929. *Macacus philippinensis* 7, obtained in Manila, was injected subcutaneously with 0.75 cubic centimeter of a suspension prepared by macerating twenty-two infected *A. ægypti*, lot 3, in 2 cubic centimeters of physiologic salt solution. There was no elevation of temperature. The leukocyte counts dropped from 10,050 before inoculation to about 7,000 on the fifth, sixth, and seventh days, which was not considered significant.

Experiment 11.—February 28, 1929. A long-tailed monkey of unknown origin (No. 8), brought to the Philippine Islands by a sailor, was obtained in Manila from the Bureau of Science. This monkey was bitten by five infected *A. ægypti*, lot 2. There was no elevation of temperature. The leukocyte counts, which had fluctuated between 14,700 and 17,450 before inoculation, were 9,150 and 8,850 the sixth and seventh days. However, in view of the great normal daily fluctuations noted later, these observations were not considered to be significant of dengue.

Experiment 12.—February 28, 1929. A long-tailed monkey of unknown origin (No. 9), brought to the Philippine Islands several years ago by a sailor, was obtained from the Bureau of Science. This monkey was bitten by eleven infected *A. ægypti* of lot 2. No evidence of dengue was observed by physical examination or from the daily temperature reaction. The preliminary leukocyte counts varied from 12,350 to 17,650. The lowest count obtained from this monkey was 7,900 cells on the fourth day after inoculation. This was not regarded as significant.

Experiment 13.—April 20, 1929. *Macacus philippinensis* 7 was inoculated intracardially with 2 cubic centimeters of citrated blood from a typical but unproved case of dengue (F.V. 18–P). No evidence of dengue was observed in this monkey during a period of nine days. April 25, 2 cubic centimeters of blood was drawn from the heart of monkey *M. philippinensis* 7 and injected intracardially into monkey 9. May 2, seven days later, 3 cubic centimeters of citrated blood from the heart of monkey 9 was inoculated intracardially into *M. philippinensis* 14. May 7, five days later, 2 cubic centimeters of citrated blood from monkey 14 was inoculated intracardially into *M. philippinensis* 16, and May 11, 2 cubic centimeters of citrated blood from *M. philippinensis* 4 was again inoculated into monkey 16 and 2 cubic centimeters into *M. philippinensis* 17. May 11, 2 cubic centimeters of citrated blood from the heart of monkey 16 was injected intrapleurally into *M. philippinensis* 18. The six monkeys used in this series of tests all failed to show any significant indication of dengue infection.

Experiment 14.—April 29, 1929. *Macacus philippinensis* 10 and 11, obtained in Manila, were each inoculated intracardially with 2 cubic centimeters of infective blood, which had just been collected from an experimental case of dengue (A.V. 10–H) during the first day of the disease. There was no apparent change in the physical appearance of this monkey. However, the temperature of monkey 11 reached 104° F. on the fifth day, and on the sixth day the neutrophilic granulocytes and lymphocytes of this monkey were markedly reduced. Monkey 11 showed nothing of significance with the possible exception of a reduction in neutrophiles after the fourth day. These reactions were not considered diagnostic.

Experiment 15.—May 1, 1929. *Macacus philippinensis* 12 and 13, obtained in Manila, were each injected intracardially with 5.5 cubic centimeter amounts of citrated blood immediately after its collection from an experimental case of dengue (A.V. 8–G), during the first day of the disease. The daily temperature and leukocyte reactions failed to show any significant changes.

Experiment 16.—May 14, 1929. *Macacus philippinensis* 20 and 21, both very young, were obtained in Manila. Each was injected subcutaneously with 2 cubic centimeters of citrated blood drawn from an experimental case of dengue (A.V. 9–D) during the first day of the disease. May 15, each received an additional 2 cubic centimeters of citrated blood from (A.V. 9–D) subcutaneously. Neither showed any physical evidence of illness, and the temperature and leukocyte counts were irregular. The lowest count for monkey 20 was 11,500, obtained the fourth day; and the lowest for monkey 21 was 7,600, obtained the same day. There was no recognizable sign of dengue.

Experiment 17.—May 19, 1929. *Macacus philippinensis* 19 was inoculated intracardially with 3 cubic centimeters of citrated blood, collected from the heart of *M. philippinensis* 21, the fifth day after that monkey had been inoculated with infected blood. No significant change was noted in the appearance of No. 19 or in the temperature or leukocyte counts.

Practically all of the monkeys used in these seventeen experiments were later reinoculated with materials known to contain dengue virus. The results were not significant, and the variations in temperature and leukocyte counts were irregular, as in the initial tests.

COMMENT

1. In these experiments twenty-six monkeys, including four *Macacus rhesus* from Washington, D. C., one *M. fuscatus* from Tokyo, and twenty-one *M. philippinensis* from an endemic dengue area (Manila), were inoculated with infective blood from experimental dengue-fever patients or from monkeys showing some slight indication of dengue infection, or with mosquitoes containing dengue virus, or were bitten by dengue-infected mosquitoes.

2. Before and after the inoculations these animals were observed daily for physical signs of illness, and daily records were made of the rectal temperature and of the leukocyte counts.

3. While some of the inoculated animals showed a decrease in the numbers of leukocytes that was suggestive, these changes

were not sufficiently striking or constant to be considered as diagnostic of infection with dengue.

II. DENGUE PRODUCED IN MONKEYS AND PROVED BY RETRANSFER OF THE INFECTION THROUGH MOSQUITOES TO MAN[4]

The failure of the monkeys to show typical physical signs and the leukopenia observed during dengue in man seemed to indicate either that they were completely immune to dengue or that the infection was manifested in some way not recognizable by our examinations. However, as a few of the animals, for example, *Macacus philippinensis* 1 and 2, had blood counts that were at least suggestive of a leukopenia on the fourth, fifth, or sixth day after inoculation; and as this corresponds roughly with the incubation period in man, it was decided to test the animals for infectivity, regardless of symptoms, by feeding normal mosquitoes on them during the fifth or sixth day after inoculation, and later allowing these mosquitoes to feed on man.

EXPERIMENTS IN WHICH INOCULATED MONKEYS WERE TESTED FOR DENGUE VIRUS ON EITHER THE FIFTH OR THE SIXTH DAY

MACACUS RHESUS

Experiment 1.—January 9, 1929. *Macacus rhesus* 1, brought to Manila in a screened cage from Washington, D. C., was inoculated subcutaneously with 1 cubic centimeter of citrated blood, which had just been drawn from an experimental dengue patient (A.V. 2–M), during the second day of the disease. This monkey showed no recognizable physical signs of dengue. However, the leukocyte curve showed some depression below the level of the preliminary counts made before inoculation. This was particularly true of the neutrophilic granulocytes, which were decreased in numbers on the fourth, fifth, and sixth days following the inoculation; while the numbers of lymphocytes increased steadily from about 3,500 cells January 9 to 10,000 January 16.

Attempt to infect normal mosquitoes.—January 15, or the sixth day after inoculation, fourteen normal *A. ægypti* took blood from this monkey and were designated lot 4. Ten mosquitoes of this lot were later combined with two of lot 5, and nine of lot 6, both of which had fed on other inoculated monkeys.

Test of mosquitoes for infectivity.—February 12 all the mosquitoes in the combined lots fed on a susceptible volunteer (A.V. 3–A), but dengue did not result.

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[4] During the progress of these studies Blanc et al. (14) (1929) reported that they were successful in transmitting dengue virus from man to two species of monkeys (*Cynomolgus fascicularia* and *Cercopithicus callitricus*) and back to man by blood inoculations. They were unable to transmit the infection from monkey to monkey. They apparently did not attempt to transmit dengue from monkey to man by mosquitoes.

TABLE 36.—*Total leukocytes and temperature records of monkeys following inoculations of dengue virus.*

Experiment No.	Monkey No.	Date of experiment.	White blood count and temperature.	Days following inoculation.								
				0	1	2	3	4	5	6	7	8
1 (a)	M.R. 2	Jan. 10, 1929	Total W.B.C	15,050	15,600	12,450	10,400	22,850	12,650	11,800	8,100	11,700
			Temperature °F	101.0	100.1	99.9	100.2	100.1	100.1	101.1	100.8	101.0
				102.4	102.0	102.2	--------	--------	100.0	101.4	100.0	99.2
1 (b)	M.R. 2	Apr. 20, 1929	Total W.B.C	--------	--------	10,800	14,100	9,500	13,050	8,100	9,450	10,900
			Temperature °F	--------	--------	100.8	100.8	101.6	101.6	100.8	102.8	101.6
2 (a)	M.R. 3	Jan. 31, 1929	Total W.B.C	14,450	10,650	10,250	12,100	10,100	8,950	7,350	8,700	10,100
			Temperature °F	100.1	100.2	101.6	99.9	101.2	100.4	101.2	101.0	101.2
				--------	100.6	100.4	--------	101.6	100.6	99.8	100.4	99.4
2 (b)	M.R. 3	Apr. 20, 1929	Total W.B.C	--------	--------	7,250	8,600	8,550	8,850	8,750	9,350	7,750
			Temperature °F	--------	--------	102.0	101.2	103.0	102.6	102.6	103.4	102.6
3	M.R. 4	Jan. 31, 1929	Total W.B.C	35,650	17,450	11,300	13,450	10,400	8,500	12,750	9,050	9,200
			Temperature °F	102.0	101.2	100.2	101.6	102.2	102.1	101.4	101.6	101.8
				--------	100.6	100.8	--------	101.8	101.8	100.6	100.8	99.6
4	M.R. 5	Feb. 28, 1929	Total W.B.C	12,100	11,100	10,200	13,150	9,400	16,350	22,100	28,950	29,800
			Temperature °F	99.8	99.2	99.4	100.0	99.4	99.0	99.4	101.4	100.8
5 (a)	M.F. 1	Mar. 1, 1929	Total W.B.C	9,900	8,150	9,150	12,850	13,100	15,850	15,900	11,150	12,200
			Temperature °F	100.8	102.6	101.2	102.2	101.2	101.8	101.8	101.6	101.0
5 (b)	M.F. 1	Apr. 20, 1929	Total W.B.C	--------	--------	12,150	5,150	9,350	7,850	7,100	6,650	5,350
			Temperature °F	--------	--------	102.0	102.0	102.4	102.2	102.0	103.0	102.4
6 (a)	M.P. 1	Dec. 17, 1928	Total W.B.C	--------	8,400	7,950	8,150	6,500	5,200	6,500	6,550	6,700
			Temperature °F	--------	99.8	99.1	98.9	98.8	99.2	100.1	99.0	98.9
B-2	M.P. 3	Jan. 9, 1929	Total W.B.C	10,850	9,100	13,300	11,050	8,900	9,250	7,350	6,850	6,250
			Temperature °F	99.6	98.6	99.8	99.8	99.8	98.9	99.1	99.2	98.9
				--------	99.8	99.0	99.4	--------	--------	99.2	99.0	101.6
7 (a)	M.P. 2	Dec. 17, 1928	Total W.B.C	12,050	11,900	9,100	8,650	5,050	5,150	10,100	8,650	7,900
			Temperature °F	--------	99.9	100.0	99.8	99.1	99.9	101.6	98.6	99.1

B-3	M.P. 5	Jan. 10, 1929	Total W.B.C.	15,600	18,950	16,200	9,850	22,250	12,100	12,400	6,350	4,350
			Temperature °F	97.2 100.2	99.9 99.6	99.2 102.2	98.9 -------	99.1 -------	98.7 100.0	98.8 99.2	98.4 99.2	98.8 97.8
8	M.P. 4	Jan. 10, 1929	Total W.B.C.	11,050	12,850	14,950	7,300	12,450	7,150	7,100	7,800	9,250
			Temperature °F	99.1 100.0	98.9 100.0	100.2 99.8	98.8 -------	99.1 -------	98.9 99.4	98.9 99.8	98.8 100.2	98.4 99.8
9	M.P. 6	Jan. 31, 1929	Total W.B.C.	32,300	7,700	10,100	6,950	10,850	15,250	13,950	17,450	13,850
			Temperature °F	99.1 -------	98.9 100.0	98.3 99.8	98.4 -------	99.2 99.8	98.8 98.6	98.8 99.2	98.6 99.4	98.8 99.4
10	M.P. 7	Jan. 31, 1929	Total W.B.C.	10,050	12,150	7,850	9,850	8,900	7,150	7,850	7,050	9,150
			Temperature °F	98.8 -------	98.4 99.4	98.6 99.2	100.0 -------	98.4 99.2	98.4 98.8	98.2 99.4	98.0 99.0	98.4 99.2
11	M.P. 8	Feb. 28, 1929	Total W.B.C.	17,450	10,950	12,100	15,050	11,800	12,400	9,150	8,850	13,100
			Temperature °F	101.0	101.8	101.4	99.8	99.8	100.8	99.2	100.2	100.2
12	M.P. 9	Feb. 28, 1929	Total W.B.C.	12,350	15,450	13,850	14,350	7,900	14,450	10,800	10,250	16,850
			Temperature °F	102.6	98.8	100.2	99.8	100.2	101.2	101.0	101.2	101.2
13 "A"	M.P. 7	Apr. 20, 1929	Total W.B.C.	-------	-------	12,150	6,850	7,800	8,200	7,950	7,800	6,400
			Temperature °F	-------	-------	101.8	101.8	101.0	100.0	100.8	101.8	102.2
13 "B"	M.P. 9	Apr. 25, 1929	Total W.B.C.	15,450	15,400	18,350	13,900	14,050	12,400	10,300	9,500	10,600
			Temperature °F	101.4	101.4	100.8	100.6	101.0	100.8	101.2	101.0	101.0
14 "A"	M.P. 10	Apr. 29, 1929	Total W.B.C.	-------	19,700	23,200	18,100	14,950	13,000	11,950	18,250	12,200
			Temperature °F	-------	100.8	101.4	101.6	102.8	102.6	102.2	102.2	102.0
14 "B"	M.P. 11	Apr. 29, 1929	Total W.B.C.	-------	15,650	23,450	12,350	15,800	11,500	10,350	9,600	11,900
			Temperature °F	-------	101.2	102.4	102.6	103.4	103.6	104.0	103.2	102.4
15 "A"	M.P. 12	May 1, 1929	Total W.B.C.	-------	8,050	14,350	9,800	12,450	14,050	10,350	10,300	11,300
			Temperature °F	-------	98.2	100.4	99.8	101.2	100.2	99.2	100.0	99.2
15 "B"	M.P. 13	May 1, 1929	Total W.B.C.	-------	10,150	18,300	14,650	15,100	19,650	14,400	15,650	15,450
			Temperature °F	-------	101.2	100.4	101.0	101.4	101.0	101.2	100.2	101.4
13 "C"	M.P. 14	May 2, 1929	Total W.B.C.	-------	11,200	11,900	13,400	10,700	7,900	10,850	9,600	8,300
			Temperature °F	-------	102.8	102.2	102.4	101.6	101.6	102.0	101.4	100.6
13 "D"	M.P. 16	May 7, 1929	Total W.B.C.	-------	13,100	15,550	8,400	11,000	11,950	10,950	10,650	13,200
			Temperature °F	-------	101.4	102.0	101.8	101.0	101.0	101.6	101.6	101.0
13 "E"	M.P. 17	May 11, 1929	Total W.B.C.	12,650	13,900	11,250	15,400	10,300	16,600	11,100	12,350	11,800
			Temperature °F	102.0	100.6	101.2	101.8	102.0	101.4	101.6	102.0	102.2

TABLE 36.—*Total leukocytes and temperature records of monkeys following inoculations of dengue virus*—Continued.

Experiment No.	Monkey No.	Date of experiment.	White blood count and temperature.	Days following inoculation. 0	1	2	3	4	5	6	7	8
13 "F"	M.P. 18	May 11, 1929	Total W.B.C.	15,850	21,450	20,800	20,800	30,700	22,600	17,150		
			Temperature °F	102.2	102.4	102.0	103.0	102.8	102.6	103.2		
17	M.P. 19	May 19, 1929	Total W.B.C.		16,650	12,300	12,550	16,750	10,800	8,100	9,700	13,100
			Temperature °F		101.8	100.8	100.6	100.2	100.0	100.4	100.0	100.0
16 "A"	M.P. 20	May 14, 1929	Total W.B.C.		24,300	15,100	13,500	11,950	11,400	16,650	16,700	12,400
			Temperature °F		101.0	101.4	100.2	102.6	102.2	103.0	102.4	101.6
16 "B"	M.P. 21	May 14, 1929	Total W.B.C.	12,950	9,900	14,600	11,550	7,750	9,650	12,400	9,200	12,000
			Temperature °F	102.2	101.8	101.4	101.6	101.0	101.8	101.6	102.2	101.4

Experiment No.	Monkey No.	Date of experiment.	White blood count and temperature.	Days following inoculation. 9	10	11	12	13	14	15	16
1 (a)	M.R. 2	Jan. 10, 1929	Total W.B.C.	11,600							
			Temperature °F	100.8							
1 (b)	M.R. 2	Apr. 20, 1929	Total W.B.C.	13,000	10,300	10,400					
			Temperature °F	101.8	101.0	102.0					
2 (a)	M.R. 3	Jan. 31, 1929	Total W.B.C.	8,800		8,050					
			Temperature °F	100.8		101.4					
2 (b)	M.R. 3	Apr. 20, 1929	Total W.B.C.	7,000	6,850	7,700					
			Temperature °F	103.0	102.4	102.2					
3	M.R 4	Jan. 31, 1929	Total W.B.C.	10,400		10,900					
			Temperature °F	101.2		102.2					

4	M.R. 5	Feb. 28, 1929	Total W.B.C.	31,050	29,050	22,250	26,050	33,600			
			Temperature °F	101.8	100.8	98.8	98.4	98.2			
5 (a)	M.F. 1	Mar. 1, 1929	Total W.B.C.	10,800	11,400	16,150	14,850	12,650	9,850	10,100	9,650
			Temperature °F	100.2	101.4	99.0	99.2	99.2	100.2	99.2	99.0
5 (b)	M.F. 1	Apr. 20, 1929	Total W.B.C.	7,850	9,050	9,550	12,900	10,500	12,900	12,450	12,550
			Temperature °F	102.8	102.2	102.0	102.0	102.2	102.4	102.4	102.0
6 (a)	M.P. 1	Dec. 17, 1928	Total W.B.C.	6,250	6,900	9,000	7,650				
			Temperature °F	100.1	99.2	99.2	99.1				
B-2	M.P. 3	Jan. 9, 1929	Total W.B.C.	17,850	25,450						
			Temperature °F	99.3 98.4	98.8						
7 (a)	M.P. 2	Dec. 17, 1928	Total W.B.C.	8,300	8,100	7,700	6,950				
			Temperature °F	99.2	99.4	100.4	100.3				
B-3	M.P. 5	Jan. 10, 1929	Total W.B.C.	8,600							
			Temperature °F	99.2							
8	M.P. 4	Jan. 10, 1929	Total W.B.C.	11,250							
			Temperature °F	98.6							
9	M.P. 6	Jan. 31, 1929	Total W.B.C.	17,150		13,450					
			Temperature °F	101.0		100.8					
10	M.P. 7	Jan. 31, 1929	Total W.B.C.	6,850		7,150					
			Temperature °F	99.2		98.6					
11	M.P. 8	Feb. 28, 1929	Total W.B.C.	11,450	12,250	8,800	8,650	11,300	12,150	12,800	
			Temperature °F	99.0	100.2	99.2	99.0	98.8	99.0	101.0	
12	M.P. 9	Feb. 28, 1929	Total W.B.C.	15,100	14,100	18,650	22,550	8,800			
			Temperature °F	99.4	100.6	102.4	102.0	102.2			
13 "A"	M.P. 7	Apr. 20, 1929	Total W.B.C.	7,450	7,900	10,250	10,750	14,250	19,700	23,400	29,950
			Temperature °F	102.0	101.4	101.8	101.4	101.8	102.6	102.8	102.6
13 "B"	M.P. 9	Apr. 25, 1929	Total W.B.C.	16,050	10,000	14,050	14,250	15,050	16,600		
			Temperature °F	101.8	101.4	101.0	100.0	101.4	101.8		

TABLE 36.—*Total leukocytes and temperature records of monkeys following inoculations of dengue virus*—Continued.

Experiment No.	Monkey No.	Date of experiment.	White blood count and temperature.	Days following inoculation.							
				9	10	11	12	13	14	15	16
14 "A"	M.P. 10	Apr. 29, 1929	Total W.B.C.	11,850	11,350	--------	--------	--------	--------	--------	--------
			Temperature °F	101.6	102.4	--------	--------	--------	--------	--------	--------
14 "B"	M.P. 11	Apr. 29, 1929	Total W.B.C.	9,000	10,600	--------	--------	--------	--------	--------	--------
			Temperature °F	103.6	103.0	--------	--------	--------	--------	--------	--------
15 "A"	M.P. 12	May 1, 1929	Total W.B.C.	9,400	12,400	9,200	10,300	16,250	--------	--------	--------
			Temperature °F	99.6	99.6	99.0	98.6	98.8	--------	--------	--------
15 "B"	M.P. 13	May 1, 1929	Total W.B.C.	20,500	15,600	13,600	12,200	11,400	--------	--------	--------
			Temperature °F	99.4	100.4	100.0	100.2	99.6	--------	--------	--------
13 "C"	M.P. 14	May 2, 1929	Total W.B.C.	7,850	7,650	9,350	11,750	13,350	9,750	10,900	8,650
			Temperature °F	101.6	101.0	101.0	101.4	100.2	101.6	102.0	101.0
13 "D"	M.P. 16	May 7, 1929	Total W.B.C.	17,400	16,250	14,850	--------	--------	--------	--------	--------
			Temperature °F	101.8	101.4	101.4	--------	--------	--------	--------	--------
13 "E"	M.P. 17	May 11, 1929	Total W.B.C.	12,900	19,050	22,200	--------	--------	--------	--------	--------
			Temperature °F	100.6	102.0	101.8	--------	--------	--------	--------	--------
13 "F"	M.P. 18	May 11, 1929	Total W.B.C.	--------	--------	--------	--------	--------	--------	--------	--------
			Temperature °F	--------	--------	--------	--------	--------	--------	--------	--------
17	M.P. 19	May 19, 1929	Total W.B.C.	11,250	8,800	--------	--------	--------	--------	--------	--------
			Temperature °F	100.4	101.2	--------	--------	--------	--------	--------	--------
16 "A"	M.P. 20	May 14, 1929	Total W.B.C.	16,150	19,700	--------	--------	--------	--------	--------	--------
			Temperature °F	101.8	101.4	--------	--------	--------	--------	--------	--------
16 "B"	M.P. 21	May 14, 1929	Total W.B.C.	12,500	--------	--------	--------	--------	--------	--------	--------
			Temperature °F	101.0	--------	--------	--------	--------	--------	--------	--------

Comment.—If dengue occurred in this monkey, the infection was not demonstrable by our observations; and on the sixth day the virus was not present in sufficient concentration to infect *A. ægypti*, lot 4. It is, of course, possible that had normal mosquitoes been allowed to feed during other days they might have become infected.

MACACUS PHILIPPINENSIS

Experiment 2.—January 9, 1928. *Macacus philippinensis* 3 (a), obtained from the Bureau of Science in Manila, was injected subcutaneously with 1 cubic centimeter of citrated blood, collected one hour previously from an experimental case of dengue (A.V. 2–M), during the second day of the disease. The rectal temperature remained normal for ten days. However, the leukocyte curve showed some depression on the fifth, sixth, and seventh days.

Attempt to infect normal mosquitoes.—January 15, or the sixth day after the inoculation, five normal *A. ægypti* were allowed to feed on this monkey, and were designated lot 5.

Test of mosquitoes for infectivity.—February 12 two of the lot 5 mosquitoes were combined with ten and nine mosquitoes, respectively, of lots 4 and 6, both of which had fed on other inoculated monkeys, and were tested on a susceptible human volunteer (A.V. 3–A). Dengue did not result from the feeding of these mosquitoes.

Comment.—Although this monkey showed no clinical evidence of dengue, and the normal mosquitoes fed on it during the sixth day after inoculation did not become infected, it is still possible that dengue occurred and that had mosquitoes been fed on other days they might have become infected.

Experiment 3.—January 10, 1929. *Macacus philippinensis* 5 (c), obtained from the Bureau of Science in Manila, was given a subcutaneous injection of 1 cubic centimeter of citrated blood drawn from an experimental case (A.V. 1–H) on the first day of the disease. No evidence of dengue was noted.

Attempt to infect normal mosquitoes.—January 15, five days later, fifteen normal *A. ægypti* were fed on the monkey and designated lot 6.

Test of mosquitoes for infectivity.—February 12 nine of these mosquitoes, with two of lot 5 and ten of lot 4, were tested by their feeding on a susceptible human volunteer (A.V. 3–A), who failed to develop dengue.

Comment.—In this experiment there was no significant clinical indication of dengue; and normal *A. ægypti* fed on the monkey on the fifth day failed to transmit the disease. However, it is possible that if normal mosquitoes had fed during other days they might have become infected.

Experiment 4.—April 14, 1930. *Macacus philippinensis* 33 (Sagada D) that had been caught in a nonendemic area in the mountains of northern Luzon, shipped to Manila in a screened box, and observed in Manila for ten days in a screened cage, was bitten by eighteen known-infected *A. albopictus* of lot 183. This monkey showed no signs of dengue.

Attempts to infect normal A. ægypti.—April 20, six days later, one hundred fifty-five normal *A. ægypti* fed on this monkey and were designated lot 214. Immediately, after the mosquitoes of lot 214 had completed their feeding, the monkey was autopsied, and the liver, spleen, and brain were removed. Forty-eight normal *A. ægypti* were fed from a feeding cell on 0.5 gram of the spleen emulsified in 2 cubic centimeters of ascitic fluid and 2 cubic centimeters of normal defibrinated human blood, and were designated lot 219. One hundred thirteen normal *A. ægypti* fed from a feeding cell on 0.5 gram of the liver emulsified in 2 cubic centimeters of ascitic fluid and 2 cubic centimeters of defibrinated normal human blood, and were designated lot 220. One hundred two normal *A. ægypti* fed from a feeding cell on 0.5 gram of the brain from monkey 33 and 0.5 gram of brain from monkey 34 emulsified in 2 cubic centimeters of ascitic fluid and 2 cubic centimeters of defibrinated normal human blood and were designated lot 218.

Test of mosquitoes for infectivity.—(*a*) May 10, 1930, seventy-six mosquitoes of lot 214 fed on susceptible volunteer A.V. 96–H, but did not cause dengue. (*b*) May 10, thirteen mosquitoes of lot 219 and thirty-nine mosquitoes of lot 220 fed on susceptible volunteer A.V. 97–II, but did not cause dengue. (*c*) See experiment 5 for testing of lot 218.

Comment.—The blood, brain, liver, and spleen of *Macacus philippinensis* 33–D were shown not to be infected with dengue virus on the sixth day following the feeding of known-infective mosquitoes on the monkey.

Experiment 5.—April 14, 1930. *Macacus philippinensis* 34 (Sagada E), that had been caught in a nonendemic area and kept screened subsequently, was bitten by nine known-dengue-infected *A. albopictus*, lot 183. No symptoms of dengue were observed in this monkey.

Attempts to infect normal A. ægypti.—April 20. This monkey was treated in the same manner as described for *M. philippinensis* 33 in experiment 4 above. Sixty-five normal *A. ægypti* fed from the living monkey and were designated lot 215. One hundred nine normal *A. ægypti* fed on spleen-ascitic-fluid-blood emulsion, and were designated lot 216. Twenty-three normal *A. ægypti* fed on liver-ascitic fluid emulsion and were designated lot 217. One hundred two normal *A. ægypti* fed from a brain-ascitic-fluid-blood emulsion and were designated lot 218.

Test of mosquitoes for infectivity.—(*a*) May 10, twenty-four mosquitoes of lot 215 fed on A.V. 98–R, but did not cause dengue. This volunteer was later found to be immune to dengue. (*b*) May 10, thirty-six mosquitoes of lot 216, five from lot 217, and forty-nine from lot 218 fed on susceptible volunteer A.V. 95–D, but did not cause dengue.

Comment.—*Macacus philippinensis* 34–E apparently did not contain transmissible dengue virus in the organs or blood on the sixth day following the potential infection of this monkey.

Because of the failure to demonstrate infection in these five experiments, in which attempts were made to infect mosquitoes from monkeys on the fifth or sixth days only after inoculation, at which time there was usually some decrease in the numbers of leukocytes, it was considered possible that the time selected might have corresponded with the period of extreme leukopenia observed in man, which is often too late in the disease for the infection of mosquitoes. It was also considered possible that the mosquitoes might have been fed too soon. Consequently, the following experiments were planned, in which monkeys were inoculated with dengue virus and were subsequently tested for infection by feeding different groups of normal mosquitoes on them daily between the third and the ninth days. The various groups of mosquitoes were then combined and tested for infectivity by feeding them later on susceptible volunteers.

EXPERIMENTS IN WHICH MONKEYS WERE TESTED FOR DENGUE VIRUS ON THE THIRD, FOURTH, FIFTH, SIXTH, SEVENTH, AND EIGHTH DAYS AFTER INOCULATION WITH INFECTIVE MATERIALS

MACACUS FUSCATUS

Experiment 1.—October 7, 1929 (noon). *Macacus fuscatus* 2 (W), which had been shipped from Tokyo, Japan, in a screened cage, was received in Manila September 27, 1929, and observed in a mosquito-proof cage at the Bureau of Science for ten days. October 7 this monkey was bitten by forty-four dengue-infected *A. ægypti* of lot 52, which had taken blood from an experimental case of dengue (A.V. 31-D) at 11.45 a. m., August 31, during the first day of the disease; and had been proved infected by the transmission of dengue to several susceptible human volunteers (A.V. 46-L, A.V. 44-F, A.V. 41-F, and A.V. 43-D). Daily observations were made of the monkey for a period of one month, but typical physical signs of dengue were not observed. There was some elevation of the rectal temperature from the fourth to the eighth day after infection, but as the temperature was also elevated at intervals during the period of subsequent observation, its significance remains in doubt. The total leukocyte counts, which had varied from a low of 13,600 to a high of 20,600 during the period of normal observation, dropped to 9,850, 10,050, 9,300, and 10,800 on the third, fourth, fifth, and sixth days, respectively, following the infective biting, and after a temporary rise to 17,300 on the seventh day returned to 10,300 on the eighth. This change in the total numbers of leukocytes was due to roughly similar changes in both the neutrophilic granulocytes and in the lymphocytes. On the fifth and sixth days large vacuolated monocytes, such as are frequently seen in the blood of human cases of dengue were noted. However, there was no definite leukopenia or other diagnostic evidence of dengue.

Transfer of virus from the monkey to normal Aëdes.—Beginning October 10, or the third day after the infective biting, different groups of thirty normal *A. ægypti* each were given an opportunity to feed on the monkey daily until October 15. The numbers and designations assigned to the mosquitoes that took blood from the monkey on the different days were as follows: Third day, thirty mosquitoes called lot 82; fourth day, 28 mosquitoes called lot 83; fifth day, twenty-nine mosquitoes called lot 84; sixth day, twenty-four mosquitoes called lot 85; seventh day, twenty-eight mosquitoes called lot 88; and eighth day, twenty-seven mosquitoes called lot 89.

Tests of mosquitoes for infectivity.—Test (*a*). October 28, or thirteen days after the last lot had fed on the monkey, the mosquitoes remaining alive in the various lots were all given an opportunity to feed on a single normal susceptible volunteer (A.V. 53-C), who had previously been ob-

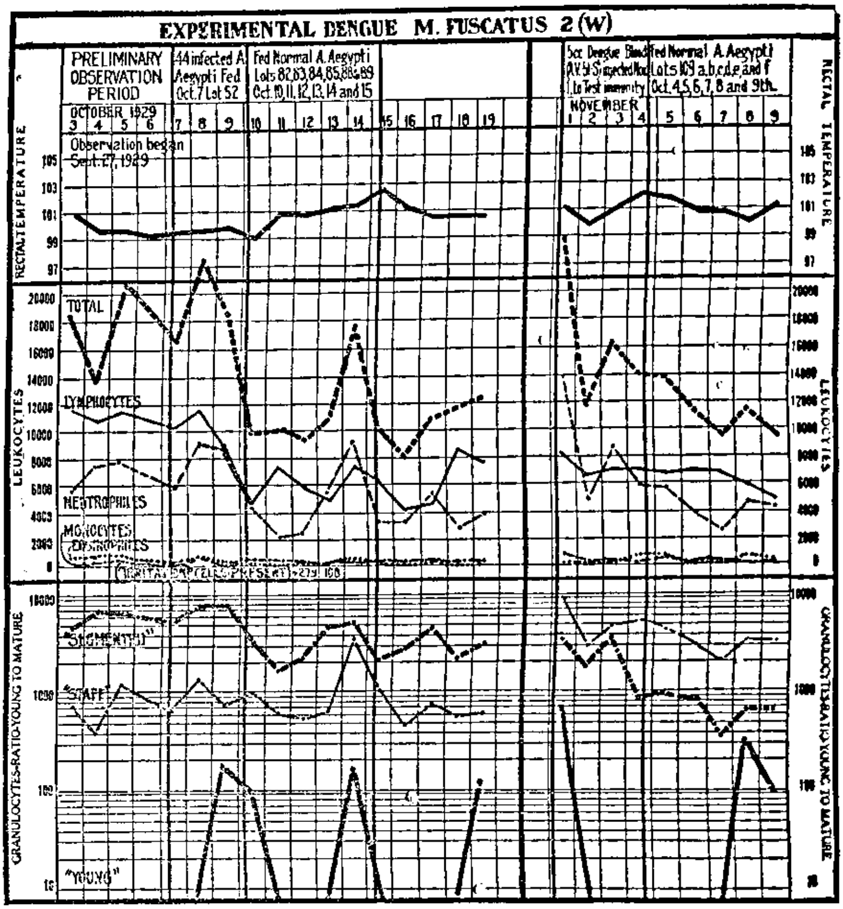


FIG. 53. Experimental dengue in *Macacus fuscatus* 2–W.

served in a screened cubicle for ten days. Six, fifteen, four, six, fifteen, and nine mosquitoes, respectively, of the various lots took blood from this volunteer who after an incubation period of seven days, developed typical dengue fever November 4 (see fig. 54).

Test (*b*).—November 5, 1929, or at least twenty-one days after having taken blood from monkey 2 (W), six, twelve, three, four, twelve, and ten mosquitoes, respectively, of those remaining alive in lots 82 to 89, were combined and given an opportunity to bite a normal Japanese monkey, *M. fuscatus* 4 (Y). Twenty-seven of the forty-seven mosquitoes took blood, and in this way transmitted dengue to the second monkey as is described in experiment 3.

Test (*c*).—November 23, 1929, or at least thirty-nine days after the feeding on *M. fuscatus* 2,-W, an attempt was made to again transfer dengue with eleven mosquitoes of the combined lots (82 to 89) to a volunteer (A.V. 58–S). However, this volunteer was later shown to be immune.

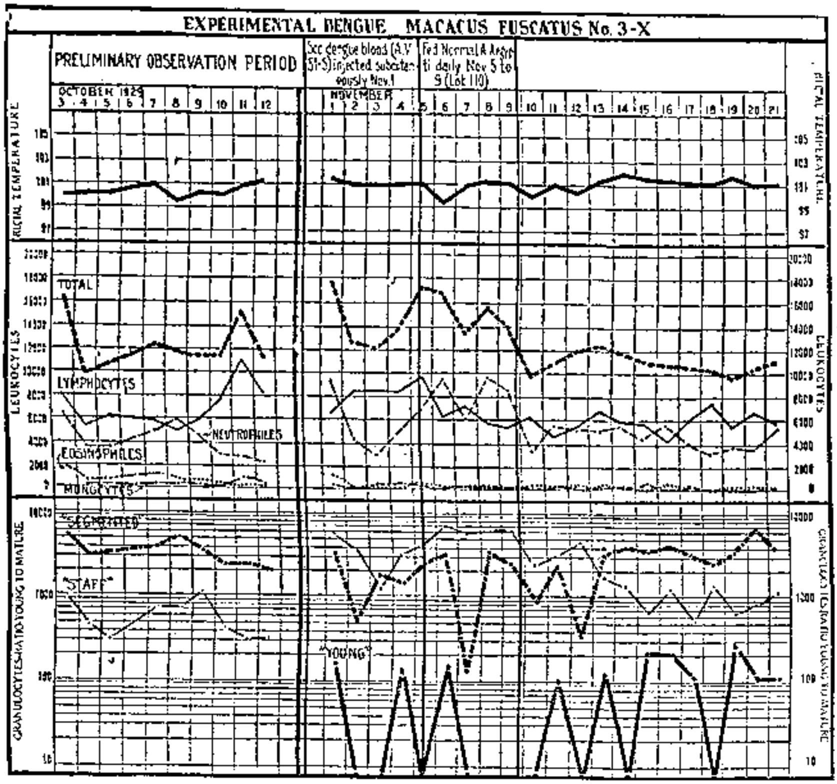


FIG. 55. Experimental dengue in *Macacus fuscatus* 3–X.

Comment.—In this experiment dengue was transmitted to a Japanese monkey (*M. fuscatus* 4 (Y), by *A. ægypti* lots 82 to 89 previously fed on *M. fuscatus* 2 (W), and from the monkey the infection was transmitted through *A. ægypti* lot 111 to man.

Experiment 4.—December 12, 1929. *M. fuscatus* 5 (Z), which had been protected from mosquitoes in screened cages since its departure from Tokyo, Japan, was bitten by eighty-four *A. ægypti* lot 110, which at least thirty-three days earlier had taken blood from *M. fuscatus* 3 (X). The monkey 5 (Z) showed no physical signs of dengue. The temperature and leukocyte counts were not significant.

Attempt to infect normal Aëdes.—Different groups of normal *A. ægypti* were fed on the fourth, fifth, and sixth days, thirty fed December 16, thirty fed December 17, and twenty-six fed December 18.

Test of mosquitoes for infectivity.—These mosquitoes were combined, designated lot 136, and given an opportunity to feed on a susceptible human volunteer (A.V. 69-K) at 9.30 a. m., January 13, 1930. Seventy mosquitoes of lot 136 took blood, but failed to produce dengue in this volunteer.

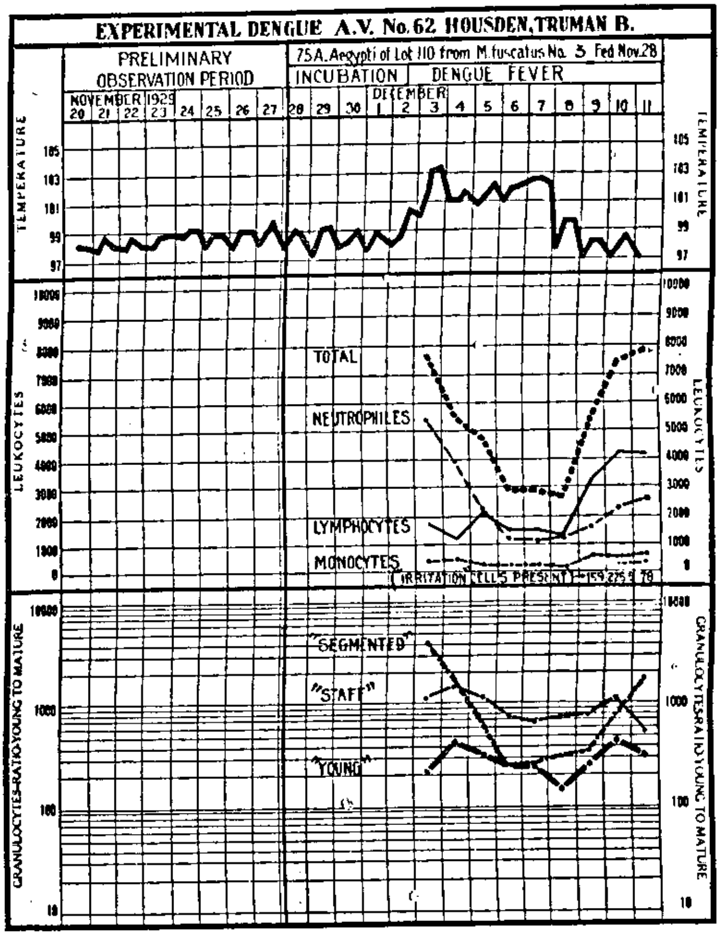


FIG. 56. Experimental dengue in American volunteer 62.

Comment.—While it is possible that this monkey, No. 5 (Z), was immune to dengue, the fact that it was tested for the presence of virus only on the fourth, fifth, and sixth days after the infectious feeding might account for the failure to prove that infection occurred.

SUMMARY

Dengue was produced experimentally in three of four Japanese monkeys (*M. fuscatus*). No significant diagnostic reactions were observed, but it was proved that the monkeys were infected by transfer of the virus through *Aëdes ægypti* to human volunteers and to monkeys.

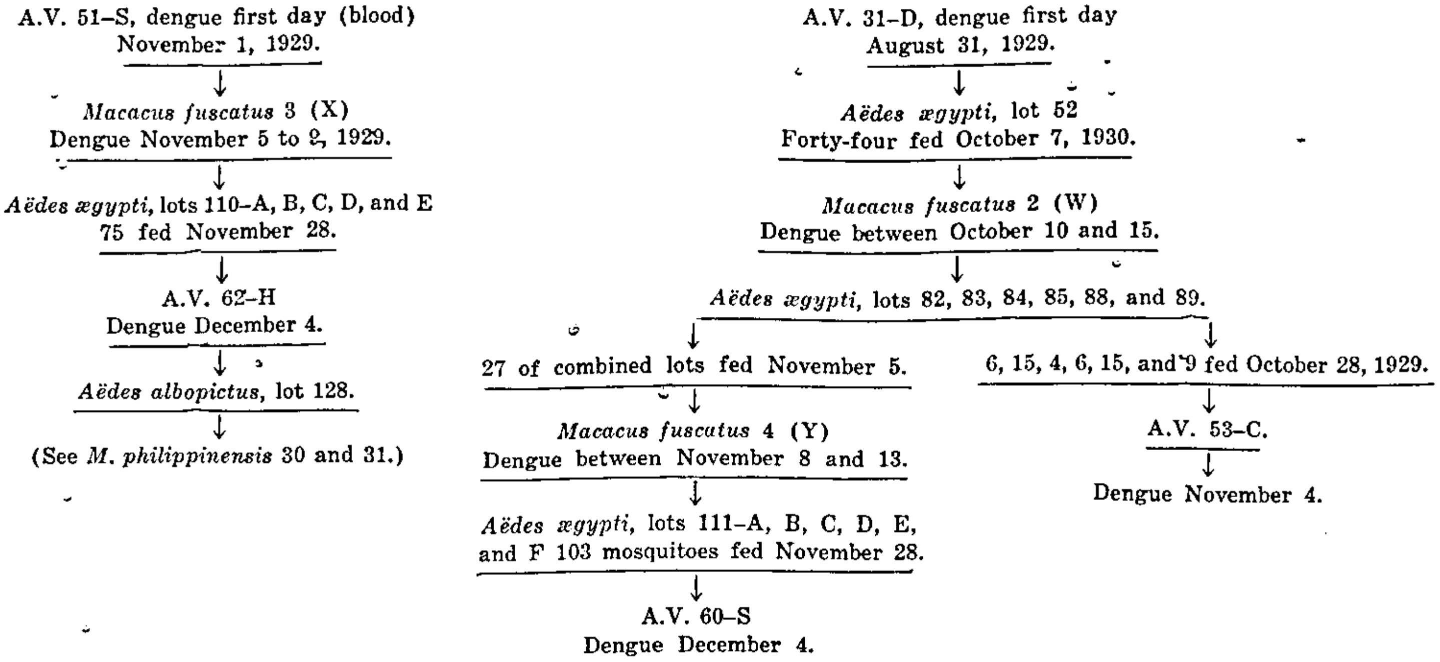

A.V. 51–S, dengue first day (blood)
November 1, 1929.
Macacus fuscatus 3 (X)
Dengue November 5 to 9, 1929.
Aëdes ægypti, lots 110–A, B, C, D, and E
75 fed November 28.
A.V. 62–H
Dengue December 4.
Aëdes albopictus, lot 128.
(See *M. philippinensis* 30 and 31.)
A.V. 31–D, dengue first day
August 31, 1929.
Aëdes ægypti, lot 52
Forty-four fed October 7, 1930.
Macacus fuscatus 2 (W)
Dengue between October 10 and 15.
Aëdes ægypti, lots 82, 83, 84, 85, 88, and 89.
27 of combined lots fed November 5.
Macacus fuscatus 4 (Y)
Dengue between November 8 and 13.
Aëdes ægypti, lots 111–A, B, C, D, E,
and F 103 mosquitoes fed November 28.
A.V. 60–S
Dengue December 4.
6, 15, 4, 6, 15, and 9 fed October 28, 1929.
A.V. 53–C.
Dengue November 4.

CHART 4.—*Transfer of dengue virus through Japanese monkeys.*

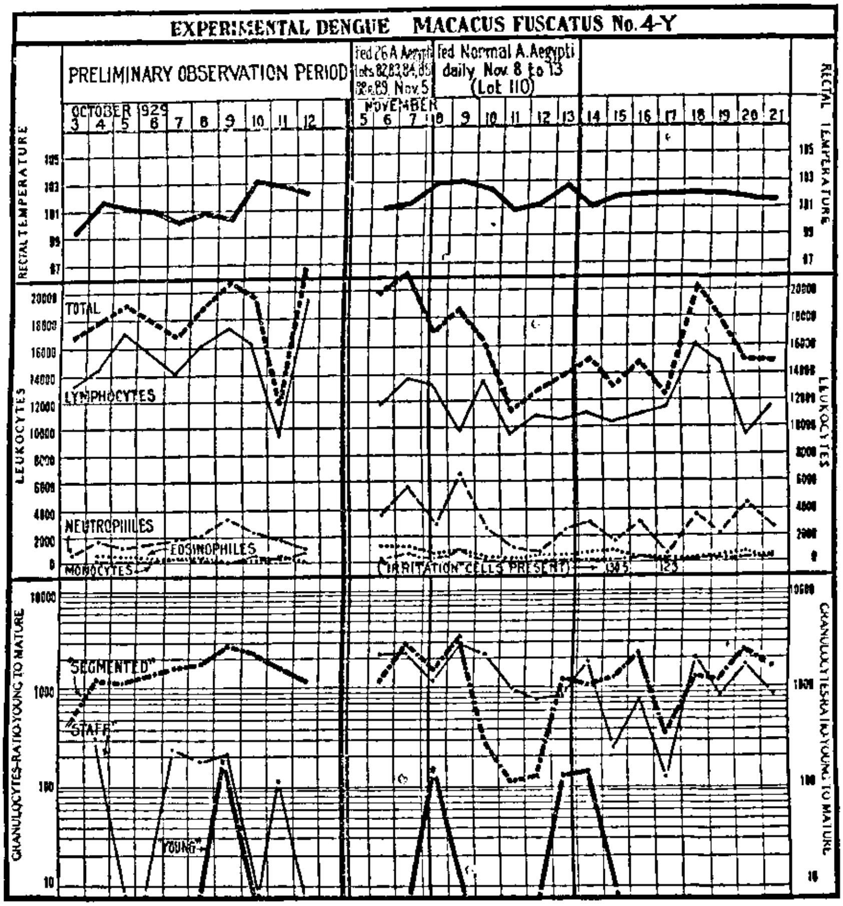


FIG. 57. Experimental dengue in *Macacus fuscatus* 4-Y.

MACACUS PHILIPPINENSIS

Seven monkeys (*Macacus philippinensis*) Nos. 22, 23, 25, 26, 27, 28, and 29, which had been kept unscreened for several years in the animal house of the Bureau of Science in Manila, were used in the following experiments:

Infective inoculations.—August 2, 1929, a suspension of twenty-five infective *A. ægypti* of lot 30, and twenty-five *A. ægypti* of lot 32 were macerated in a mortar with sterile sand, suspended in 14 cubic centimeters of physiologic salt solution, filtered through a wisp of cotton, and injected into the different monkeys as follows: *M. philippinensis* Nos. 22, 23, 27, 28, and 29 each received 1 cubic centimeter of mosquito suspension intracardially and 0.4 cubic centimeter subcutaneously; monkey 26 received 3

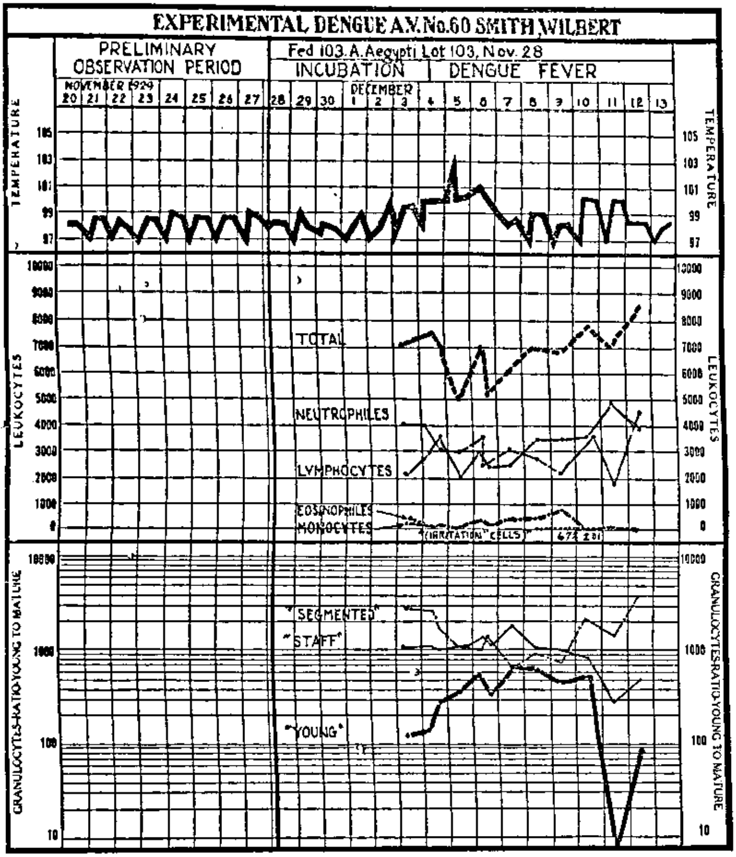


FIG. 58. Experimental dengue in American volunteer 60.

cubic centimeters intracardially and 1.5 cubic centimeters subcutaneously; while monkey 25 received 1 cubic centimeter subcutaneously.

Attempt to transfer virus from monkeys to normal Aëdes.—After these injections each monkey was exposed daily, August 5, 6, 7, 8, and 9, to different lots of mosquitoes, each composed of twenty normal *A. ægypti.* The numbers of mosquitoes which took blood each day are shown in Table 37.

As indicated in Table 37, the five groups of mosquitoes fed on each monkey were then combined in a single cage and given lot numbers as follows: The mosquitoes from monkey 22 were called lot 92–A; from monkey 23, lot 92–B; from monkey 25, lot 92–C; from monkey 26, lot

92–D; from monkey 27, lot 92–E; from monkey 28, lot 92–F; and from monkey 29, lot 92–G.

TABLE 37.—*Numbers of mosquitoes that fed on Macacus monkeys.*

Macacus philippinensis No.	Number of macerated dengue-infected *Aëdes* infected August 2, 1929.		Evidence of reaction.	Number of normal *A. ægypti* fed on monkeys August 5 to 9.					
	Intracardially.	Subcutaneously.		5	6	7	8	9	Total.
22	3.6 (1 cc)	1.4 (0.4 cc)	None	20	15	11	11	11	68 (lot 92–a).
23	3.6 (1 cc)	1.4 (0.4 cc)	do	20	16	13	9	9	67 (lot 92–b).
25		3.6 (1.0 cc)	do	16	20	14	13	11	74 (lot 92–c).
26	10.7 (3 cc)	5.4 (1.5 cc)	do	21	17	16	9	13	76 (lot 92–d).
27	3.6 (1 cc)	1.4 (0.4 cc)	do	19	17	7	15	14	72 (lot 92–e).
28	3.6 (1 cc)	1.4 (0.4 cc)	do	20	19	14	12	10	75 (lot 92–f).
29	3.6 (1 cc)	1.4 (0.4 cc)	do	14	18	14	19	13	78 (lot 92–g).

Macacus philippinensis No.	Test for infectivity of mosquitoes.			
	Number fed.	American volunteer.	Date.	Result.
22	9	46–L	September 23	Negative.
23	14	43–D	September 21	Do.
25	28	42–H	September 21	Dengue fever September 26.
26	33	41–F	September 16	Negative.
27	15	44–F	September 21	Do.
28	5	40–W	September 14	? (Immune).
29	28	45–V	September 21	Negative.

TESTS TO DETERMINE INFECTIVITY OF MOSQUITOES. LOTS 92–A, B, C, D, E, F, AND G

Experiment 5.—In order to determine whether it was worth while to test the different mosquito lots from the seven monkeys separately, lots 92–A, B, C, D, E, F, and G were first tested for infectivity as a group by giving them all an opportunity to feed on a susceptible volunteer (A.V. 35–E), who had been observed in a screened cubicle for eight days previous to the experiment. This volunteer was bitten by an unknown number of mosquitoes from each of the respective lots, and also by *A. ægypti* of lot 93, which were later proved to be free from dengue. After an incubation period of seven days the man developed a typical attack of dengue. Comment: The occurrence of dengue in A.V. 35–E indicated that one or more of the monkeys (22, 23, 25, 26, 27, 28, or 29) had been infected with dengue, and that the infection had been transmitted to man by some of the mosquitoes tested.

Experiment 6.—In order to confirm the results obtained in test "a," seventeen, twenty-four, thirty-seven, thirty-six, twenty-nine, twenty-three,

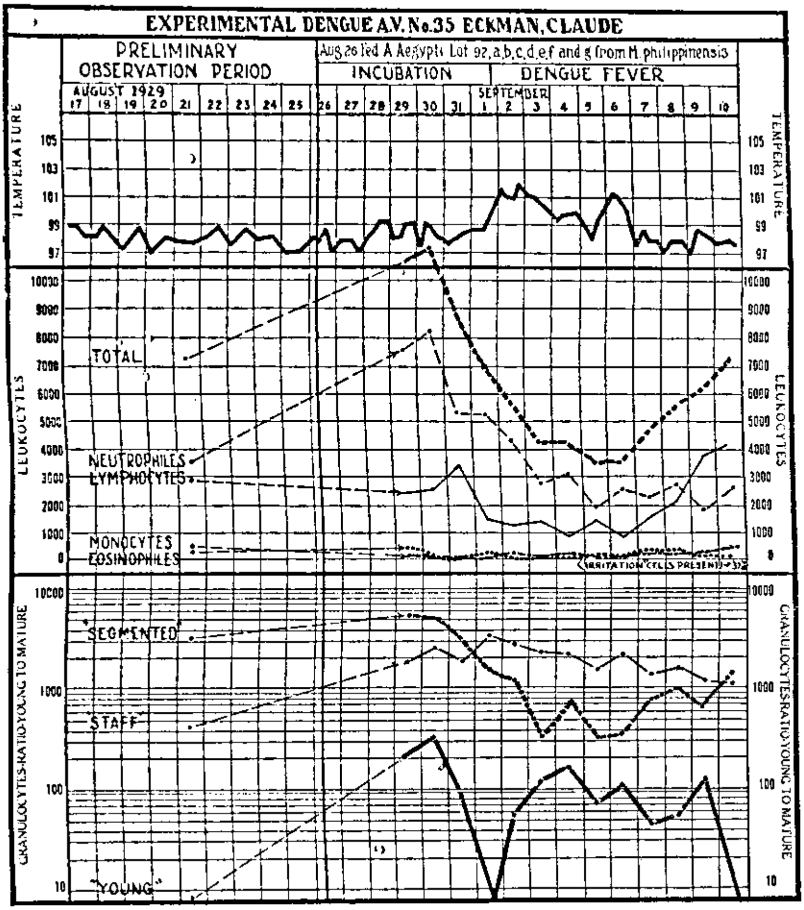


FIG. 59. Experimental dengue in American volunteer 35.

and thirty-seven *A. ægypti* of the respective lots, were again tested for infectivity September 5 by feeding them on another susceptible volunteer (A.V. 38–H). After an incubation period of five days this volunteer also developed typical dengue fever. Comment: This second positive test confirmed the first and proved that one or more of the monkeys had been infected with dengue.

In order to determine the number of monkeys infected, the following tests were made in which each of the seven lots of *A. ægypti* (92–A, B, C, D, E, F, and G) were fed separately on a different human volunteer.

Experiment 7.—Aëdes ægypti of lot 92–A from *M. philippinensis* 22. September 23, 1929, nine *A. ægypti* which remained alive of the sixty-eight of lot 92–A which at least forty-five days earlier had fed on monkey 22, took blood this date from a susceptible volunteer (A.V. 46–L), As dengue

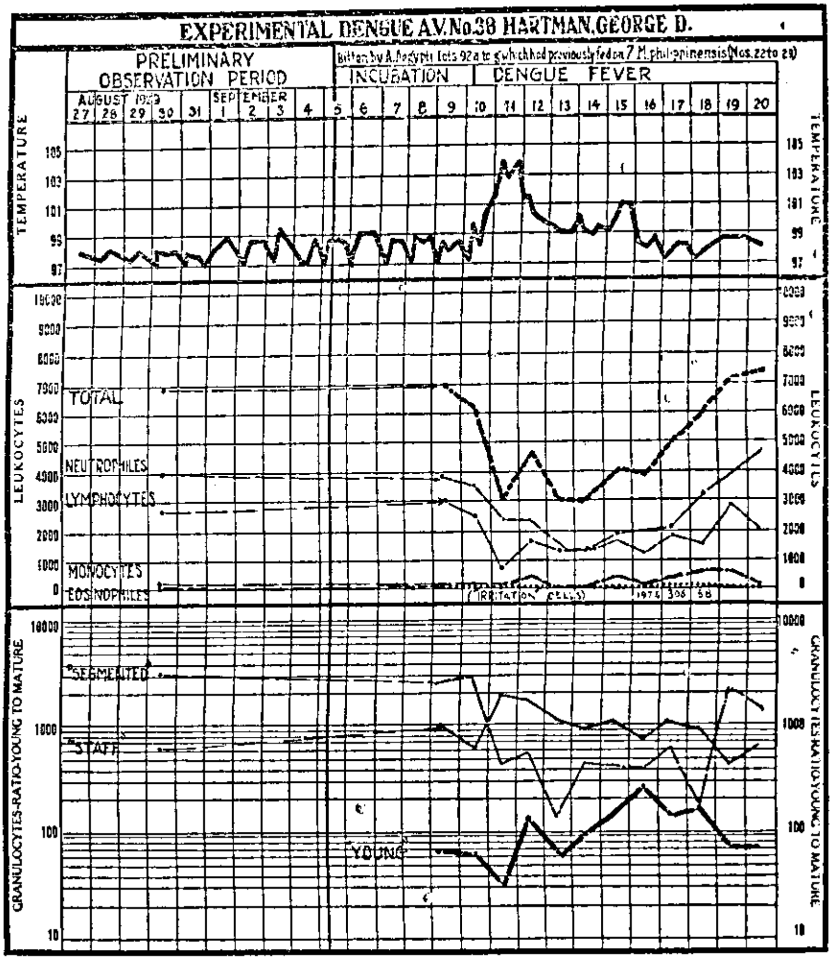


FIG. 60. Experimental dengue in American volunteer 38.

did not result from this feeding, it is apparent that the remaining mosquitoes of lot 92–A were not infected. While lot 92–A may have originally contained infected mosquitoes that had died before this test, there is no proof that this occurred. There was no indication that *M. philippinensis* 22 had been infected with dengue.

Experiment 8.—Aëdes ægypti, lot 92–B, from *M. philippinensis* 23. September 21, 1929, fourteen *A. ægypti* which remained alive of the sixty-seven of lot 92–B which had fed at least forty-three days earlier on monkey 23, took blood this date from a susceptible volunteer (A.V. 43–D). Since this man failed to develop dengue during the usual incubation period, it is apparent that the mosquitoes used of lot 92–B had not been infected by feeding on *M. philippinensis* 23. There was no evidence to prove that monkey 23 had been infected with dengue.

Experiment 9.—Aëdes ægypti, lot 92–C, from *M. philippinensis* 25. September 21, 1929. Twenty-eight of the *A. ægypti* of lot 92–C, which remained of the seventy-four that had fed at least forty-three days earlier on monkey 25, took blood this date from a susceptible volunteer (A.V. 42–H). That some of these mosquitoes were infected was proved by the fact that after an incubation period of five days this volunteer developed typical dengue fever.

Experiment 10.—Aëdes ægypti, lot 92–D, from *M. philippinensis* 26. September 16, 1929 (noon). Thirty-three *A. ægypti* of lot 92–D, which remained of the seventy-five that had fed thirty-eight or more days earlier on monkey 26, fed this date on a susceptible volunteer (A.V. 41–F). Dengue did not result.

Experiment 11.—Aëdes ægypti, lot 92–E, from *M. philippinensis* 27. September 21, 1929, 9 a. m. Fifteen of the original seventy-two *A. ægypti* of lot 92–E, which had taken blood forty-three or more days earlier from monkey 27, fed this date on a susceptible volunteer (A.V. 44–F). Dengue did not develop from this feeding.

Experiment 12.—Aëdes ægypti, lot 92–F, from *M. philippinensis* 28. September 14, 1929. Five of the original seventy-five *A. ægypti* of lot 92–F, which had taken blood thirty-six days earlier on monkey 28, fed this date on a volunteer (A.V. 40–W). Dengue did not result from this feeding, but since this volunteer was later shown to be immune the infectivity of lot 92–F mosquitoes remained in doubt.

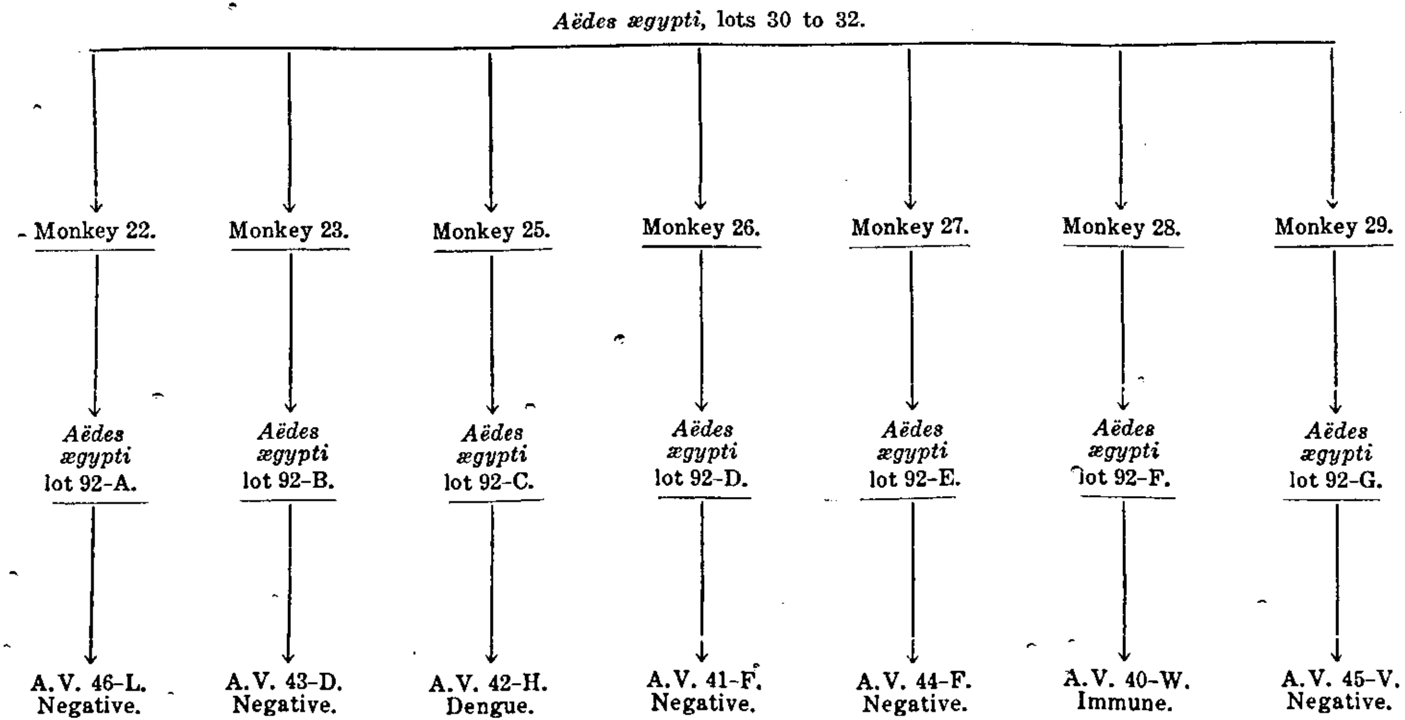
Experiment 13.—Aëdes ægypti, lot 92–G, from monkey 29. September 21, 1929, 9 a. m. Twenty-eight of the original seventy-eight *A. ægypti* of lot 92–G, which had taken blood at least forty-three days previously from monkey 29, were fed this date on a susceptible volunteer (A.V. 45–V). Dengue did not develop from this feeding. There was no proof that monkey 29 had dengue.

As it was suspected that a large proportion of *M. philippinensis* raised in localities where dengue is not endemic might be susceptible to the infection, monkeys were obtained from Mountain Province in northern Luzon in which region dengue does not occur. Three monkeys were caught at an elevation of about 4,000 feet, near Sagada, Philippine Islands, and were transported to Manila in boxes screened with 20-mesh wire netting. After periods of normal observation in mosquito-proof cages these three monkeys were used in the following dengue transmission experiments:

Experiment 14.—January 16, 1930, *M. philippinensis* 30 (Sagada A) was bitten by thirty-two infected *A. albopictus* of lot 128, which forty-three days previously had taken blood from an experimental case (A.V. 62–H) during the first day of dengue. The monkey failed to show any recognizable clinical evidence of infection.

However, normal *A. ægypti* were fed on the monkey, as follows: Twenty-five January 20, twenty-nine January 21, thirty January 22, twenty-five January 23, and twenty-seven January 24.

CHART 5.—*Diagrammatic chart indicating attempts to pass dengue virus through Macacus philippinensis monkeys obtained from an endemic dengue area of the Philippines.*



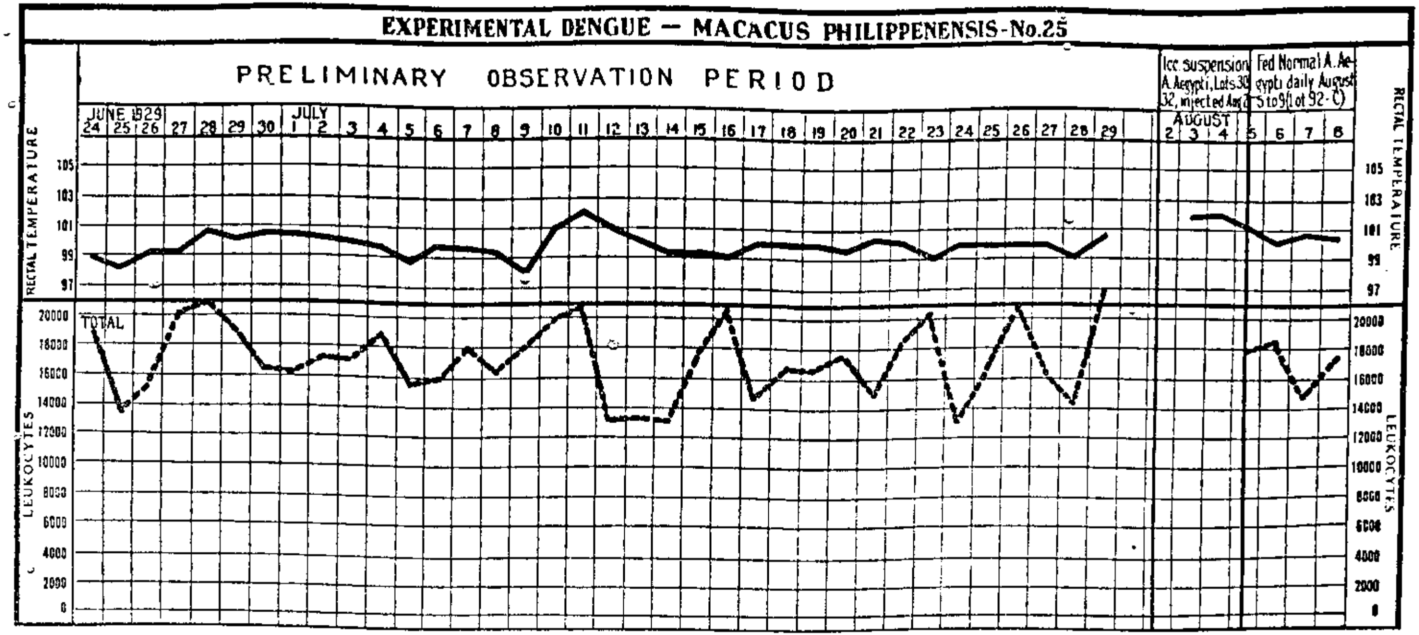


FIG. 61. Experimental dengue in *Macacus philippinensis* 25.

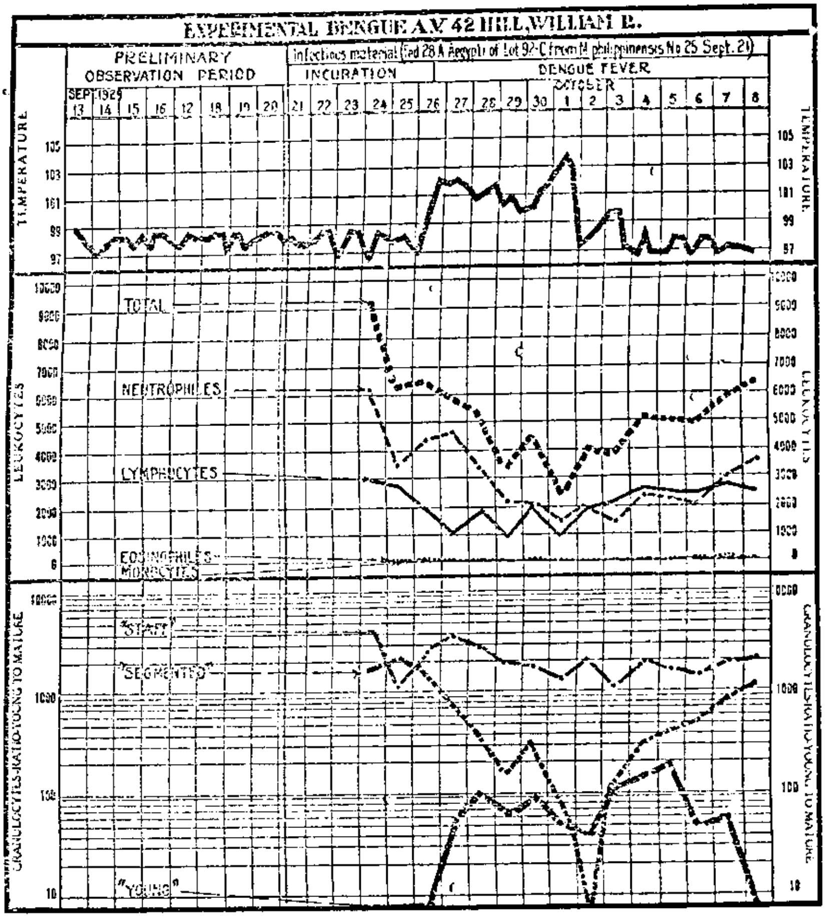


FIG. 62. Experimental dengue in American volunteer 42.

February 15, 1930 (10.30 a. m.) seventy-three of these five groups of mosquitoes, which had been combined and designated lot 156, were fed on a susceptible volunteer (A.V. 80-L). After an incubation period of six days the volunteer developed typical dengue fever.

February 24, 1930, an attempt was made to transmit dengue from monkey to monkey by feeding thirty-one *A. ægypti* of lot 156 on *M. philippinensis* 32 (Sagada C), but dengue was not proved in the latter monkey as is described in experiment 16.

Experiment 15.—January 16, 1930, *M. philippinensis* 31 (Sagada B) was bitten by thirty-five infected *A. albopictus* of lot 128, which forty-three days previously had taken blood from an experimental case of dengue (A.V. 62-H), during the first day of the disease. The monkey failed to show any recognizable clinical evidence of infection. Normal *A. ægypti* were fed on the monkey as follows: Twenty-seven January 20, thirty

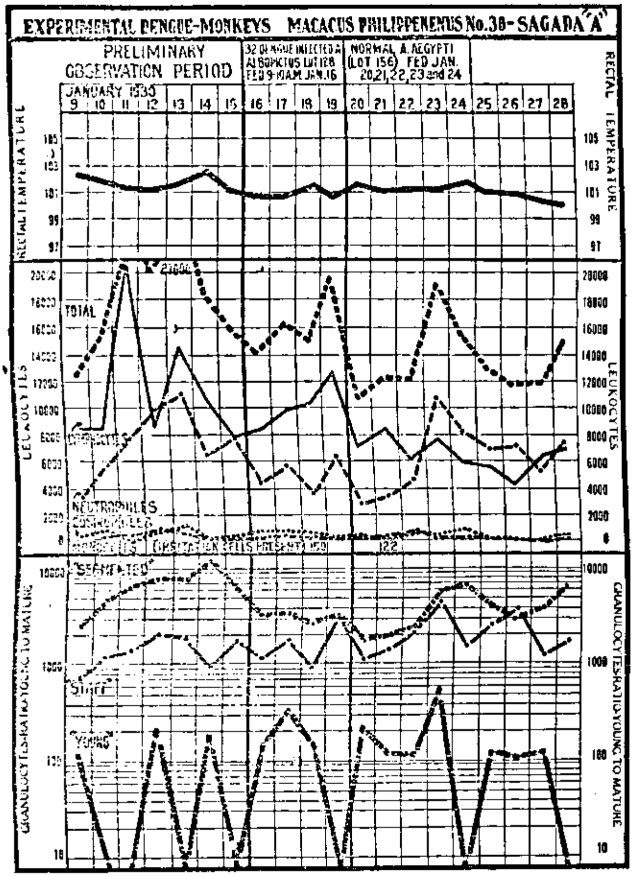


FIG. 63. Experimental dengue monkeys *Macacus philippinensis* 30–Sagada "A."

January 21, twenty-six January 22, twenty-nine January 23 and thirty-one January 24. February 15 eighty-seven mosquitoes of these combined groups, which had been designated lot 157, were tested for infectivity by feeding on a susceptible volunteer (A.V. 81–G). After an incubation period of five days, this volunteer developed typical dengue fever.

Experiment 16.—February 24, 1930, *M. philippinensis* 32 (Sagada C) was bitten by thirty-one of the one hundred thirty-six *A. ægypti* remaining in lot 156, which had at least thirty-one days earlier fed on *M. philippinensis* 30 (Sagada A). The infectivity of some of the mosquitoes of lot 156 had been proved earlier by their transmission of dengue to a human volunteer. The monkey (32–C) was tested for dengue by feeding on it groups of normal *A. ægypti* as follows: Twenty-seven February 28, thirty-three March 1, thirty-three March 2, twenty-six March 3, and twenty-

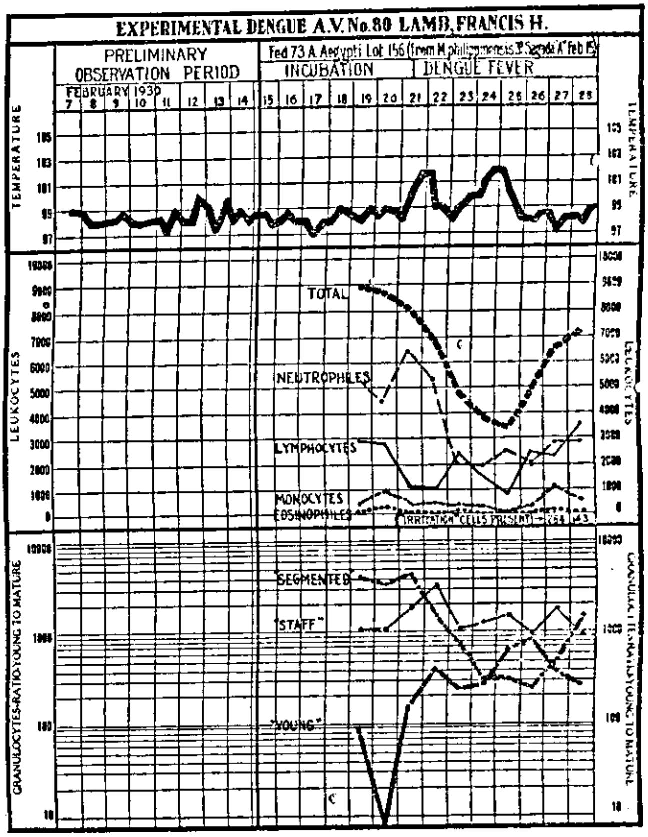


FIG. 64. Experimental dengue in American volunteer 80.

nine March 4. March 22 (11 a. m.) seventy-six of these combined groups of mosquitoes, called lot 172, fed on a susceptible volunteer (A.V. 90-A), who failed to develop dengue. Comment: There was no evidence to indicate the occurrence of dengue in *M. philippinensis* 32 (Sagada C). It is, of course, possible that the infected mosquitoes in lot 156 had all died before feeding on this monkey, but there is no evidence to prove that this occurred.

DISCUSSION

Studies of daily temperature reactions and leukocyte counts on more than forty-one monkeys, inoculated with dengue virus, indicate that neither of these tests was of assistance in the diagnosis of dengue in these animals. The rectal temperature of apparently normal monkeys was found to vary considerably from day to day, and as a rule the temperature curves for the periods after inoculation showed similar daily variations. The numbers of leukocytes in blood collected from monkeys be-

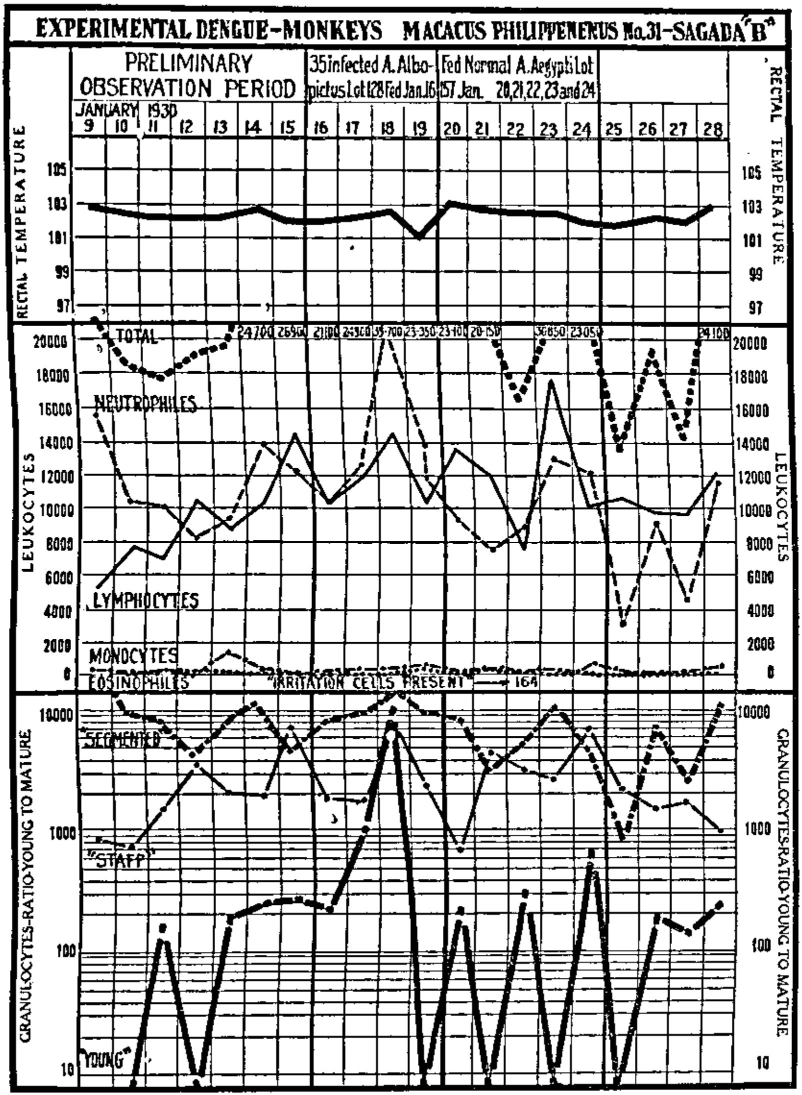
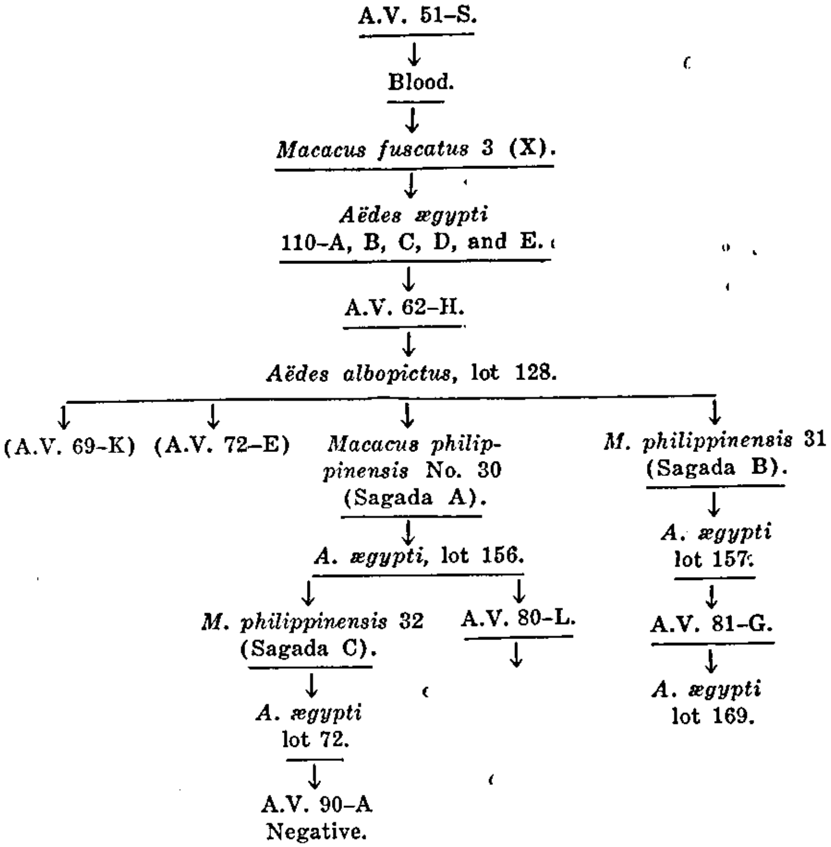


FIG. 65. Experimental dengue monkeys *Macacus philippinensis* 31-Sagada "B".

fore inoculation were also irregular, and the counts were often extremely high. Preliminary observations on normal monkeys showed that markedly different leukocyte counts were obtained in blood collected simultaneously from the ear, from the sole of the foot, and from the finger of a single animal, and for this

CHART 6.—*Indicating transfer of dengue virus to and from Philippine monkeys obtained from a dengue-free location.*


A.V. 51-S.
Blood.
Macacus fuscatus 3 (X).
Aëdes ægypti 110-A, B, C, D, and E.
A.V. 62-H.
Aëdes albopictus, lot 128.
(A.V. 69-K)
(A.V. 72-E)
Macacus philippinensis No. 30 (Sagada A).
M. philippinensis 31 (Sagada B).
A. ægypti, lot 156.
A. ægypti lot 157.
M. philippinensis 32 (Sagada C).
A.V. 80-L.
A.V. 81-G.
A. ægypti lot 72.
A. ægypti lot 169.
A.V. 90-A Negative.

reason most of the counts in these experiments were made in blood from a finger or toe. Whether due to technical difficulties or to variations in their leukocyte counts, very little evidence was obtained of a definite leukopenia, even in the animals known to have been infected with dengue. However, it may be significant that, as has been reported by previous workers, some of the leukocyte curves showed a suggestive drop after a period corresponding to the incubation period required for the development of dengue in man. The technic employed in proving that certain of the monkeys had developed dengue has certain limitations because of the possibility that in some instances a negative result might have been obtained because the mosquitoes fed during the particular time of the monkey's infectivity might

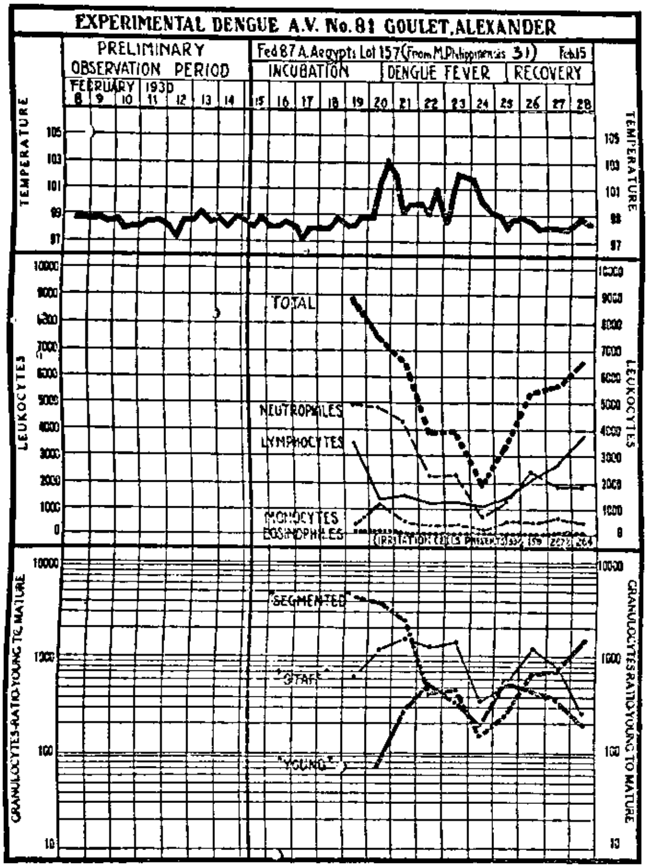


FIG. 66. Experimental dengue in American volunteer 81.

have died before the combined lots for the entire period were tested on volunteers. However, in spite of the fact that the method employed is too uncertain and tedious for routine diagnosis, it has served an important purpose in this study by furnishing proof that certain monkeys may be infected with dengue, and that the virus multiplies in their blood until it reaches concentrations sufficient to result in its transmission through *Aëdes ægypti* to other monkeys and to man. The significance of the one negative transmission experiment with *M. rhesus* must remain in doubt, since this animal was tested for infectivity only on the fifth day after the inoculation of virus. However, dengue infection was proved in three of the four monkeys from Japan (*M. fuscatus*), in one of seven monkeys (*M. philippinen-*

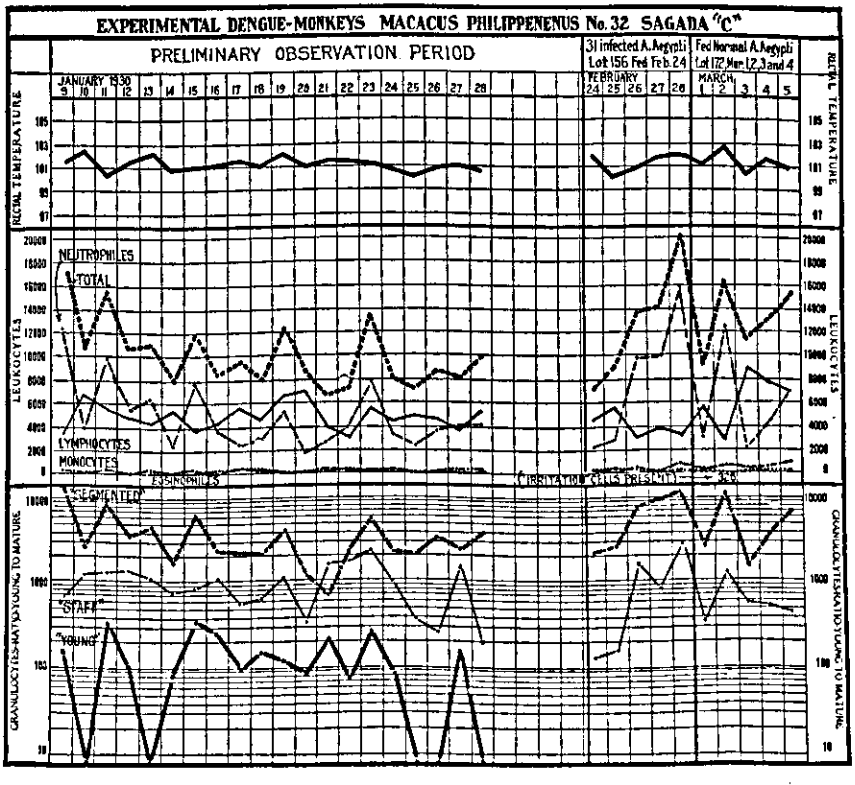


FIG. 67. Experimental dengue monkeys *Macacus philippinensis* 32–Sagada "C."

sis) obtained in or around Manila, where dengue is endemic, and in two of the three monkeys of the same species from a nonendemic area in the mountains of northern Luzon. Considering the limitations of the method used, it seems probable that monkeys other than those identified might also have been infected.

The knowledge that dengue can be transmitted by infected mosquitoes to monkeys and that from the latter the infection can be retransmitted through mosquitoes to other monkeys and to man is of considerable interest and importance because of its bearing on the epidemiology of dengue. It would appear that in certain localities the monkey may be an important factor in the spread of this desease.

The fact that susceptible individuals of a single species (*M. philippinensis*) were found both in endemic and nonendemic areas, but that a larger proportion of the former seemed resistant to infection, appears to indicate that immunity to dengue is probably acquired as a result of accidental infection at some time between birth and adult life. If this is true, it would not appear improbable for the virus of dengue to be maintained during long' periods of time, either in the jungle or in sparsely populated localities by passage through the young of monkeys alone.

These observations are paralleled by the results obtained experimentally in attempts to produce dengue in natives from different sections of the Philippines. In an earlier chapter it is shown that adult natives living in endemic areas are generally immune to dengue, while others living in the nonendemic Mountain Province of Luzon, at elevations of 4,000 to 5,000 feet, were found to be susceptible. It is apparent that monkeys, particularly in the Philippines, may be an important factor in the spread of dengue.

SUMMARY AND CONCLUSIONS

1. Ten chickens, two lizards, six mice, twelve guinea pigs, seven rabbits, and twenty-eight monkeys (*Macacus philippinensis*) which were obtained in dengue-endemic areas, were inoculated with blood from dengue patients or other monkeys, emulsions of infective mosquitoes, or were bitten by infective mosquitoes.

2. Five Japanese monkeys, *Macacus fuscatus;* five Philippine monkeys, *M. philippinensis,* from non-dengue-endemic areas; five

monkeys, *M. rhesus*, obtained from the Zoölogical Park, Washington, D. C., and three guinea pigs, born and raised in a mosquito-proof cage, were either inoculated with infective blood or were bitten by dengue-infected mosquitoes.

3. Although observations were made of the temperature and leukocyte counts daily, before and after inoculations, no consistent or typical diagnostic sign of dengue was observed, even in the monkeys that were proved to be infected with dengue.

4. However, as has been observed by others, certain of the animals developed slight changes, either in temperature reactions or in the numbers of leukocytes, about the fourth, fifth, or sixth day after inoculation, and these were at least suggestive of a specific reaction.

5. Attempts were made to determine the presence or absence of infection in chickens, guinea pigs, and monkeys by feeding normal *Aëdes* mosquitoes on them on certain arbitrarily selected days after the inoculation, and later testing the mosquitoes on susceptible human volunteers.

6. Four cockerels obtained in Manila, which previous to their experimental use were probably exposed to dengue-infected mosquitoes, were not infected with dengue, as shown by the failure of mosquitoes, fed on these chickens on the third to eighth days inclusive, to transmit dengue to a susceptible volunteer.

7. Three guinea pigs, fifteen to eighteen days of age, which from birth had been protected from mosquitoes, were tested for dengue infection by means of mosquitoes that had fed on these animals in one instance during the fourth, fifth, sixth, seventh, and eighth days and on two other guinea pigs on the fifth, sixth, seventh, and eighth days following their potential infection. The mosquitoes failed to infect susceptible volunteers.

8. In the transmission experiments with monkeys the results were as follows:

(*a*) (Animals tested for infectivity on the fifth and sixth days only.) In one *M. rhesus* from Washington, one *M. fuscatus* from Tokyo, two *M. philippinensis* from Manila, Philippine Islands, and two *M. philippinensis* from Sagada, Philippine Islands, the infection was not proved by the transfer of virus to man.

(*b*) (Animals tested for infectivity on several days between the third and eighth.) Dengue infection was produced in three of four *M. fuscatus* from Tokyo, in one of seven *M. philippinensis* from Manila, and in two of three *M. philippinensis* from Sagada, a dengue-free locality.

9. The apparently greater prevalence of susceptible animals among monkeys from localities where dengue is not prevalent seems to indicate that, as with the adult native Filipino, immunity is probably acquired as a result of natural infections during early life.

10. These observations indicate that negative conclusions should not be drawn concerning the possibility of infecting lower animals with dengue unless (*a*) the animals tested are known to have been protected throughout their lives from the bites of infected mosquitoes, and (*b*) unless they are tested daily from the fourth to the eighth or the ninth day after the introduction of the virus by transfer of blood either directly or through the mosquito vectors to susceptible volunteers.

11. It seems probable that further experiments, conducted with these requirements in mind, might show that animals other than monkeys can be infected with dengue.

12. The proof offered here establishes the fact that dengue can be transmitted to monkeys of the species *M. fuscatus* and *M. philippinensis* by infected *Aëdes;* that the virus multiplies in the blood without producing recognizable typical changes in the temperature or the leukocyte counts; that at some time between the third and the ninth day after infection, the virus may be passed to other monkeys by blood inoculation or may be transferred through *Aëdes* to other monkeys or man; and that in one monkey a single attack produced an immunity which lasted for several days.

13. Since these observations indicate that monkeys are able to take part in the natural dissemination and maintenance of virus in endemic tropical localities, they are of considerable importance to any consideration of the epidemiology of dengue.

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The various phases of these investigations were planned and carried out by the authors working coöperatively and without any sharp division of duties. Expert technical assistance was rendered by the following members of the Medical Department, three of whom had been trained for their special duties during the investigations made by Siler, Hall, and Hitchens. Staff Sergeant Jesse F. Rhodes was the assistant in general charge of the technical work and directly responsible for the breeding and handling of mosquitoes. He was aided in the mosquito breeding by Private 1st class Bonifacio Reyes (PS) (laboratory specialist, 2d class), who had been engaged in similar work during the previous six years; and by Private 1st class Gervacio Sales (PS) (laboratory specialist, 3d class), who had also been on duty with the board during the previous four years. Sergeant Carl G. Heilmann and Private 1st class Hinton F. Miller assisted in many of the miscellaneous duties connected with the work, including the experiments on animals, the work in leukocyte counts, and the preparation of charts. The board's secretaries, Mrs. Nancy Lynham Carpenter and Miss Adele Scott, assisted in the preparation of protocols, records, and manuscript. Three other members of the enlisted personnel who were not actively engaged in the work on dengue, but who helped in various ways, were Staff Sergeant Thomas Reed; Private 1st class Joseph M. Hoff, M. D. (laboratory specialist, 1st class); and Private 1st class Gregorio D. Dumlao, (PS) (laboratory specialist, 3d class).

The more technical procedures were carried out in the laboratories of the board in the Bureau of Science. Fortunately, much of the apparatus and materials formerly used by Siler, Hall, and Hitchens, and later by Schule, were still available, including a laboratory-bred strain of *Aëdes ægypti* which were descendants of the original mosquitoes used by these previous workers. In general, the methods used for breeding mosquitoes and the types of cages used for breeding and feeding them on volunteers were similar to those described in the monograph published by the former investigators.

Soldiers of the United States Army and native Ifugaos were used in the production of experimental dengue in man. When possible American volunteers were selected from groups of soldiers who had recently arrived in the Philippines. Upon admission to the ward a complete physical examination was made, including routine examinations of urine and fæces, Wassermann tests, and daily temperature and leukocyte counts. All volunteers were protected from mosquitoes by keeping them in cubicles screened with 20-mesh copper wire within a screened ward of the Sternberg General Hospital, Manila, Philippine Islands. In practically all investigations concerned with transmission or identification of the virus the volunteers were first observed in the screened cubicles for eight or more days in order to make sure that they had not been infected previous to admission to the ward.

The diagnosis of dengue in each of the experimental volunteers was established by a careful study of the symptoms, including daily temperature and leukocyte counts, and, when indicated, by subsequent transfers of the virus through mosquitoes to other volunteers. Details of the different phases of the investigation are presented as separate articles or chapters and the records of the different lots of mosquitoes and volunteers will be given in the reprint of this article in Bureau of Science Monograph 29.

ILLUSTRATIONS

PLATE 1

Aëdes albopictus Skuse.

PLATE 2

FIG. 1. Apparatus for feeding mosquitoes through skin; *a*, lid of staining dish with guinea-pig skin clamped over shallow depression which is filled with mixture of normal blood and macerated infected mosquitoes; *b*, staining dish partially filled with water heated to 55° C.

2. *Aëdes ægypti* feeding through guinea-pig skin on a mixture of normal blood and macerated dengue-infected mosquitoes.

PLATE 3

Screened cubicles for isolation of human volunteers. (Photographed by United States Army Signal Corps.)

TEXT FIGURES

FIG. 1. Annual dengue admission rates per thousand white enlisted men in the United States Army, 1928.

2. Dengue compared with all other diseases contracted by American soldiers in the Philippine Islands, 1902–1928.
3. Distribution of dengue among Army posts in the Philippine Islands, 1922–1929.
4. Dengue fever at Fort Stotsenburg, Philippine Islands.
5. Dengue at Fort Mills, Corregidor, Philippine Islands.
6. Dengue fever among troops in Manila stationed at Fort William McKinley and Camp Nichols.
7. Seasonal occurrence of dengue fever at Army posts in the Philippine Islands.
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11. Experimental dengue in American volunteer 94.
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14. Experimental dengue in American volunteer 57.
15. Experimental dengue in American volunteer 88.

16. Experimental dengue in American volunteer 47.
17. Experimental dengue in American volunteer 10.
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23. Average leukocyte counts in cases of dengue with fever of three days duration.
24. Average leukocyte counts in cases of dengue with fever of four days duration.
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27. Experimental dengue in Filipino volunteer 1.
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33. Experimental dengue in Filipino volunteer 7.
34. Experimental dengue in Filipino volunteer 8.
35. Experimental dengue in Filipino volunteer 9.
36. Experimental dengue in Filipino volunteer 10.
37. Experimental dengue in Filipino volunteer 17.
38. Experimental dengue in Filipino volunteer 18.
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41. Experimental dengue in Filipino volunteer 12.
42. Experimental dengue in Filipino volunteer 13.
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46. Experimental dengue in Ifugao volunteer 1.
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50. Experimental dengue in guinea pig 14–A.
51. Experimental dengue in guinea pig 15–B.
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53. Experimental dengue in *Macacus fuscatus* 2–W.
54. Experimental dengue in American volunteer 53.
55. Experimental dengue in *Macacus fuscatus* 3–X.
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57. Experimental dengue in *Macacus fuscatus* 4–Y.
58. Experimental dengue in American volunteer 60.
59. Experimental dengue in American volunteer 35.

60. Experimental dengue in American volunteer 38.
61. Experimental dengue in *Macacus philippinensis* 25.
62. Experimental dengue in American volunteer 42.
63. Experimental dengue monkeys *Macacus philippinensis* 30–Sagada "A."
64. Experimental dengue in American volunteer 80.
65. Experimental dengue monkeys *Macacus philippinensis* 31–Sagada "B."
66. Experimental dengue in American volunteer 81.
67. Experimental dengue monkeys *Macacus philippinensis* 32–Sagada "C."

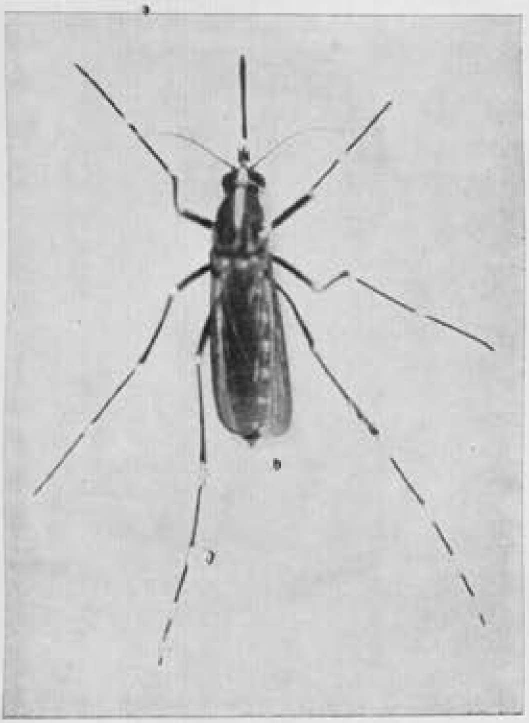
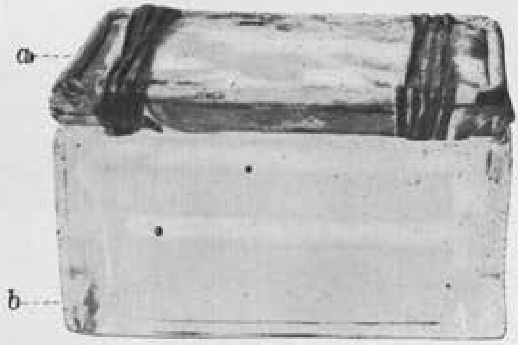


PLATE 1. AEDES ALBOPICTUS SKUSE.



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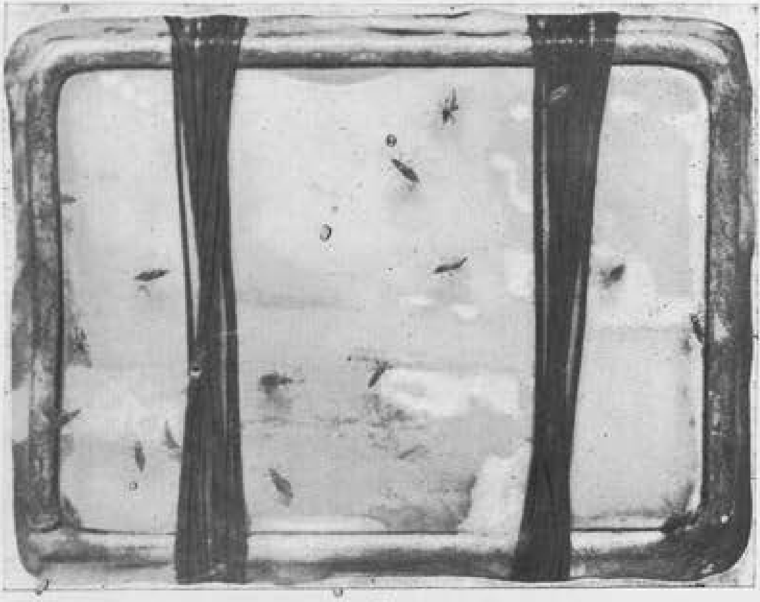

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PLATE 2. APPARATUS FOR FEEDING MOSQUITOES THROUGH SKIN.

PLATE 2.

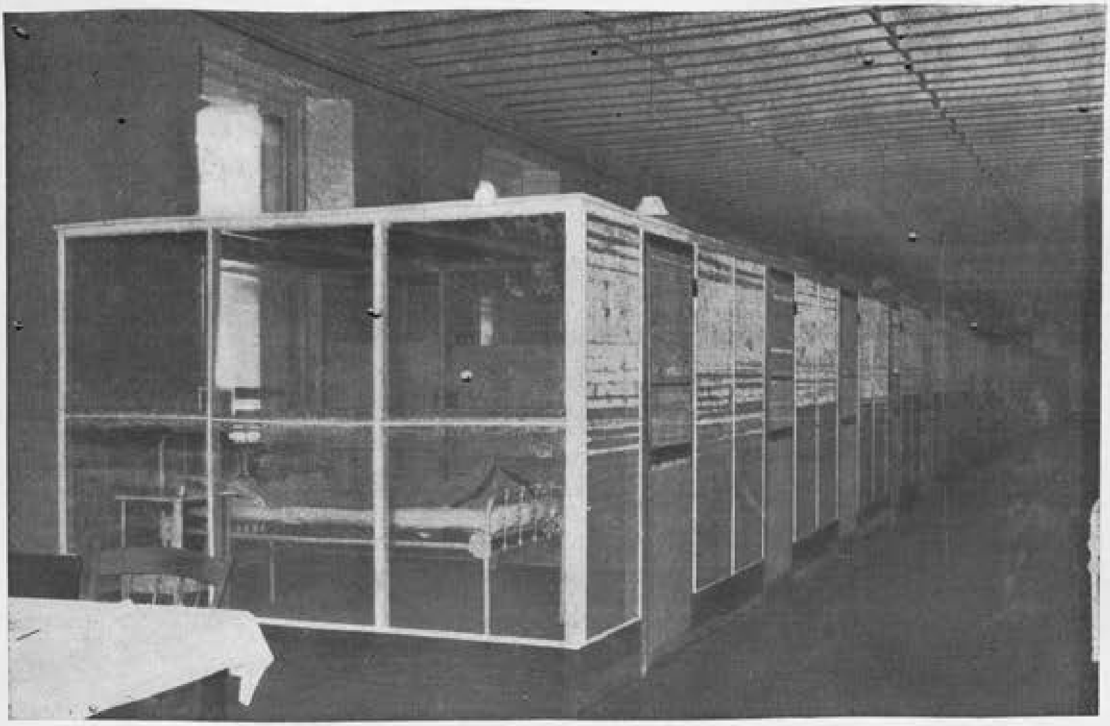

PLATE 3. SCREENED CUBICLES FOR ISOLATION OF HUMAN VOLUNTEERS.